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Possible role of the protein kinase C/CPI-17 pathway in the augmented contraction of human myometrium after gestation

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1 Activation of protein kinase C (PKC) by phorbol 12,13-dibutylate (PDBu, 1 μM) induced sustained contractions with no increase in $[Ca^{2+}]_i$ in nonpregnant and pregnant human myometria. The contractile effects of PDBu in pregnant myometrium were much greater than those in nonpregnant myometrium, and the contractions in pregnant myometrium were accompanied by an increase in myosin light chain (MLC) phosphorylation at Ser^{19}.

2 The contraction induced by PDBu in pregnant myometrium was inhibited by the inhibitors of conventional PKC isoforms, bisindolylmaleimides and indolocarbazole, such as Go6976, Go6983, and Go6850 (1 μM). LY333531 (1 μM), a specific inhibitor of PKCβ, also inhibited the PDBu-induced contraction in the pregnant myometrium.

3 In the pregnant myometrium permeabilized with α-toxin, PDBu increased the contractions induced at fixed Ca^{2+} concentration (0.3 μM) both in nonpregnant and pregnant myometria, indicating Ca^{2+} sensitization of contractile elements.

4 Western immunoblot analysis indicated that pregnant myometrium contained PKC isozymes such as conventional PKC (α, β, γ), novel PKC (δ, ε, θ), and atypical PKC (ζ but not ι and λ). RT-PCR and real-time RT-PCR analysis indicated that, among the conventional PKC, the levels of mRNA of β isoform in pregnant human myometrium were greater than those in nonpregnant myometrium.

5 CPI-17 is a substrate for PKC, and the phosphorylated CPI-17 is considered to inhibit myosin phosphatase. The levels of CPI-17 mRNA and protein expression were also greater in the pregnant myometrium.

6 These results suggest that the PKC-mediated contractile mechanism is augmented in human myometrium after gestation, and that this augmentation may be attributable to the increased activity of the β PKC isoform and CPI-17.
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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; MLC, myosin light chain; PDBu, phorbol 12,13-dibutylate; PKC, protein kinase C

Introduction

It is well known that uterotonic agonists such as oxytocin, endothelin-1, acetylcholine, and prostaglandins increase intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and muscle tone in the uterus (Anwer & Sanborn, 1989; Sakata *et al*., 1989; Sakata & Karaki, 1992; Wray, 1993; Szal *et al*., 1994; Word *et al*., 1994; Kim *et al*., 1995; Fomin *et al*., 1999; Longbottom *et al*., 2000). These receptor agonists stimulate phosphoinositide turnover in various types of smooth muscle, including uterine smooth muscle (Marc *et al*., 1986; Schrey *et al*., 1988; Schiemann *et al*., 1991), and inositol 1,4,5-trisphosphate, one of the phosphoinositide hydrolysis products, releases Ca^{2+} from intracellular Ca^{2+} stores and induces transient contractions (Somlyo & Somlyo, 1994). Another phosphoinositide product, diacylglycerol, stimulates protein kinase C (PKC), which phosphorylates various cellular functional proteins (Nishizuka, 1995; Webb *et al*., 2000). It has been reported that PKC activation with phorbol ester inhibits smooth muscle contraction by inhibiting inositol phosphate production in gastric smooth muscle (Ozaki *et al*., 1992), while it induces contraction by the activation of contractile proteins in vascular and tracheal smooth muscles (Morgan & Morgan, 1984; Sato *et al*., 1988; Ozaki *et al*., 1990). In contrast, in cultured smooth muscle cells of rat portal vein, phorbol esters increase L-type Ca^{2+} channel activity (Loirand *et al*., 1990). However, in rat myometrial cells, PKC activation inhibits Ca^{2+} channel activity (Kusaka & Sperelakis, 1995). These results suggest that the effects of PKC activation are tissue- and species-dependent, comprising both contractile and relaxing responses in which different mechanisms appear to contribute to their effects.

The rat has been widely used for the investigation of uterine biology and pharmacology. In the myometrium isolated either from pregnant or nonpregnant rat, it has been reported that

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phorbol ester induces inhibitory effects on contraction (Savineau & Mironneau, 1990; Phillippe, 1994a, b). We have also observed decreases in $[Ca^{2+}]_i$ and muscle contraction upon PKC stimulation by phorbol ester in rat myometrium, showing that PDBu inhibits the increase in $[Ca^{2+}]_i$ more strongly in myometrium from pregnant than from nonpregnant rats (Kim *et al*., 1996). In contrast to the results in rat myometrium, others have suggested the importance of PKC in the agonist-induced contractions in human myometrium (Morrison *et al*., 1996; Breuiller-Fouche *et al*., 1998; Eude *et al*., 2000). However, very little is known about the precise mechanism of PKC-induced contraction in human myometrium.

In the light of the awareness of species-related differences in PKC-mediated signaling, we attempted to examine the effects of PKC activation on $[Ca^{2+}]_i$ and contraction in myometrium isolated from pregnant and nonpregnant women. We found that PKC activation induces positive feedback control in the activation processes of the smooth muscle contractile element in human myometrium, which is distinct from findings in rat myometrium showing that PKC is involved in the negative feedback control of the contractile mechanism (Savineau & Mironneau, 1990; Kim *et al*., 1996).

Methods

Selection of patients and tissue collection

Tissue samples were obtained from the patients undergoing myomectomy, hysterectomy, and elective cesarean section. This study was approved by the University Ethics Committee (#0205) and was performed according to the Declaration of Helsinki. Written informed consent was obtained from each patient. In term delivery group, pregnant women (26–39 years of age) underwent elective lower segment cesarean section for previous cesarean section, cephalopelvic disproportion (CPD), fetal anomaly, and breech presentation at 37–40 weeks gestation. Both internal and external cervical os were found to be closed in each primigravida (nonparous women) by vaginal examination and ultrasound sonography. In multigravida (parous woman), the internal cervical os was found to be closed, although the external cervical os was opened in some cases. Additionally, they did not receive any drugs that influence uterine contractile ability, such as indomethacin or β_2 adrenoceptor stimulants. Routine cesarean section was carried out under epidural anesthesia or spinal anesthesia. After delivery of the infant and placenta, a sample of myometrium (length 2–3 cm, width 3–6 mm, thickness 4–7 mm) was taken from the upper margin of the lower uterine segment incision using tissue forceps and scissors. In the late stage of pregnancy, a transverse incision is usually made into the anterior wall of the lower uterine segment. As muscle fibers (sphincter-like fibers) around the internal cervical os are arranged transversely, the blood loss due to the transverse incision in the lower uterine segment was extremely less compared with classical longitudinal incision. The collection of myometrium from the portion of incision in the lower uterine segment was convenient and safe because extra incision was not needed. Additionally, in the cesarean section, the collection of myometrium from the portion of the unnecessary incisions was prohibited by University Ethics Committee.

Nonpregnant women (29–54 years of age) underwent either myomectomy for myoma or total abdominal hysterectomy for ovarian tumor, cervical cancer (CIS or stage 1a), endometrial cancer (stage 1a or 1b), and myoma, and the myometrium was taken at the junction of inner cervical os and uterine corpus. The obtained tissue samples were immediately submerged in ice-cold University of Wisconsin Solution (Belzer UW, Dupont Pharma, Wilmington, Netherlands) and transported to the laboratory. Tissues were used within 2 days after the dissection.

Measurement of muscle tension

Longitudinal muscle strips (width 5–6 mm, length 15–20 mm) were dissected from the isolated tissue, and were attached to a vertical holder under a resting tension of 10 mN. Preliminary experiment indicated that human myometrial tissue generated maximum active tension under a resting tension of 8–12 mN. After equilibration for 20 min in a physiological salt solution, each strip was repeatedly exposed to 40 mM KCl solution until responses became stable. The physiological salt solution contained (mM): NaCl 136.9; KCl 5.4; $CaCl_2$ 1.5; $MgCl_2$ 1.0; $NaHCO_3$ 23.8; glucose 5.5; and ethylenediaminetetraacetic acid (EDTA) 0.01. The high K^+ solution was prepared by replacing NaCl with equimolar KCl. These solutions were saturated with a 95% O_2 and 5% CO_2 mixture at 37°C and pH 7.4. Muscle contraction was recorded isometrically with a force–displacement transducer (Model TB611T, Nihon Kohden, Tokyo, Japan) connected to a Model 3134 strain amplifier and Model 3056 ink-writing recorder (Yokogawa, Tokyo, Japan). We employed the contraction induced by 40 mM K^+ at steady-state level as a reference response. In some experiments, contractile tension was expressed as absolute force (mN mg^{-1} wet weight tissue). We used only preparations that generated 40 mM K^+-induced contraction greater than approximately 5 mN.

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

$[Ca^{2+}]_i$ was measured as reported by Ozaki *et al*. (1987) with a fluorescent Ca^{2+} indicator, fura-2. Muscle strips were treated with acetoxymethyl ester of fura-2 (fura-2/AM, 5 μM) for 4–5 h at room temperature. A noncytotoxic detergent, cremophor EL (0.02%), was added to increase the solubility of fura-2/AM. After loading, the muscle strip was washed with physiological salt solution at 37°C for 20 min to remove uncleaved fura-2/AM and was held horizontally in a temperature-controlled, 7-ml organ bath. One end of the muscle strip was connected to a force–displacement transducer to monitor the muscle contraction. The muscle strip was illuminated alternately (48 Hz) at two excitation wavelengths (340 and 380 nm). The fluorescence intensity of 500 nm (F340 and F380) was measured using a fluorimeter (CAF100, JASCO, Tokyo, Japan). The ratio of F340 to F380 (F340/F380) was calculated as an indicator of $[Ca^{2+}]_i$. To assess the successful loading of fura-2, we constantly monitored F340 and F380, and only data from experiments in which F340 and F380 changed in opposite directions were used. The absolute Ca^{2+} concentration was not calculated in this experiment because the dissociation constant of the fluorescent indicator for Ca^{2+} in cytosol may be different from that obtained *in vitro* (Karaki, 1989).

Therefore, the ratios obtained in resting and high K^+-stimulated muscle were taken as 0 and 100%, respectively.

Permeabilized muscle strips were prepared by treatment with *Staphylococcus aureus* α-toxin, as described previously (Nishimura *et al*., 1988; Kitazawa *et al*., 1989). Small muscle strips, 0.1–0.2 mm in diameter and 1–2 mm in length, were made and held horizontally in a small 0.5-ml organ bath. Permeabilization was accomplished by incubating the muscle strips for 30 min with 80 $\mu g\,ml^{-1}$ α-toxin in a relaxing solution containing 130.0 mM potassium propionate, 4.0 mM $MgCl_2$, 4 mM Na_2ATP, 20.0 mM tris-maleate, 2.0 mM creatine phosphate, 10 $U\,ml^{-1}$ creatine phosphokinase, 1 mM carbonyl cyanide *p*-trifluoromethoxy-phenyldrazone, 1 mM E-64, 2 mM EGTA (pH 6.8), and indicated concentrations of free Ca^{2+}. All of the tissues were treated with 10 μM ionomycin to remove the effects of Ca^{2+} released from the sarcoplasmic reticulum. The apparent binding constant of EGTA for Ca^{2+} was considered to be 10^{-6} M at pH 6.8. Experiments were conducted isometrically at room temperature (22–24°C).

MLC phosphorylation in intact muscle

Phosphorylation of 20 kDa myosin light chain (MLC) in intact tissues was determined by Western blotting of glycerol–PAGE as described in detail previously (Sakurada *et al*., 1994). Strips of myometria were quickly frozen in dry ice–acetone containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT). The frozen muscle strips were kept on ice over 4 h, then homogenized in 10% TCA solution with 10 mM DTT. The homogenate was centrifuged at $10{,}000 \times g$ for 1 min, after which the pellet was washed with diethyl ether 4–5 times and then suspended in a urea–glycerol buffer containing 8 M urea. The suspended sample was re-centrifuged at $10{,}000 \times g$ for 5 min and the supernatant was collected. An equal amount of protein (10 $\mu g\,lane^{-1}$) was loaded on each lane of glycerol–PAGE. Glycerol–PAGE was performed to separate phosphorylated MLC, and electrophoresed proteins were blotted on PVDF membrane (Bio-Rad Lab., Hercules, CA, U.S.A.) to perform Western blotting. The antibody, prepared in our laboratory, recognizes only 20 kDa MLC phosphorylated at Ser^{19} (Sakurada *et al*., 1994). Total nonphosphorylated MLC in the same sample was measured separately, and was used as a reference MLC protein in the smooth muscle. Area of phosphorylated MLC was measured using NIH image and the results are expressed as % of the resting level (unstimulated condition). Anti-MLC antibody (polyclonal rabbit anti-bovine tracheal MLC antibody) was kindly donated by Dr J. Stull, University of Texas.

Detection of PKC isozyme and CPI-17

PKC isozymes and CPI-17 were detected by western blotting method. Equal amounts (50 μg) of protein from the myometrial tissues were loaded for analysis by 7.5–15% SDS–PAGE. The separated proteins were transferred by electrophoresis onto PVDF membrane (Biorad, Hercules, U.S.A.) and exposed to monoclonal antibodies (Transduction Laboratories, Franklin Lakes, U.S.A.) specific for the conventional PKC isozymes (PKCα, PKCβ, and PKCγ), the novel PKC isozymes (PKCδ, PKCε, PKCη, and PKCθ), the atypical PKC isozymes (PKCι and PKCλ or polyclonal anti-PKCζ) (Santa Cruz Biotechnology, Santa Cruz, U.S.A.), and CPI-17 (CPI-17(N-20) (Santa Cruz Biotechnology, Santa Cruz, U.S.A.). The membranes were incubated with a horseradish peroxidase-conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG. Detection was achieved using the enhanced chemiluminescence system following the manufacturer's instructions (Amersham Biosciences, Piscataway, U.S.A.). The immunoreactive bands were scanned with FLA-3000 (Fujifilm, Tokyo, Japan).

Semiquantitative RT–PCR

Total RNA extraction and semiquantitative RT–PCR was performed in the myometrial tissues. In the present experiments, we used the hot start method using Ampli Taq Gold DNA polymerase supplied by the Perkin-Elmer Corp. (Branchburg, NJ, U.S.A.). Total RNA was extracted from the smooth muscle strips by the acid-guanidinium isothiocyanate phenol chloroform (AGPC) method (Chomczynski & Sacchi, 1987) and the concentration of RNA was adjusted to 1 $\mu g\,\mu l^{-1}$ with RNase-free distilled water. The PCR products were electrophoresed on 2% agarose gel containing 0.1% ethidium bromide. We visualized detectable fluorescent bands with an ultraviolet (UV)-transilluminator and saved the images using FAS III (Toyobo, Tokyo, Japan).

The forward (F) and reverse (R) oligonucleotide primers for β2-microglobulin, PKCα, PKCβ, PKCγ, and CPI-17 were as follows (with suitable size for PCR products): β2-microglobulin (F: ctcacgtcatccagcagaga; R: tctttttcagtgggggtgaa; 198), PKCα (F: ggaactcaggcagaaattcg; R: cagttcttctgtgcccttcc; 196), PKCβ (F: aaattgccatcggtctgttc; R: ccttcgaattctgattggtca; 628), PKCγ (F: ttgggagaggttggagagac; R: acgaagtccgggttcacata; 189), CPI-17 (F: gacgtggagaagtggat; R: gcccggctgcttgtg; 220).

*Real-time RT–PCR analysis for PKC*β

This procedure is based on the time point during cycling when amplification of the PCR product is first detected, rather than by the amount of PCR product accumulated after a fixed number of cycles. The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above the baseline. The PKCβ target gene copy number in unknown samples is quantified by measuring Ct and by using a standard curve to determine the starting copy number.

A standard curve was constructed for the PKCβ isoform gene as the target and for the β2-microglobulin gene as an endogenous control. PCR products for PKCβ and β2-microglobulin were cloned into pCR2.1 plasmid vectors and these plasmid vector DNA diluted serially were used as standard templates. The PKCβ mRNA expression of the unknown samples was divided by the endogenous reference (β2-microglobulin) amount to obtain a normalized target value.

All PCR reactions were performed in an ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems). The reaction mixture contained the Taqman universal master mix (Perkin-Elmer Applied Biosystems), 4 μl cDNA, 260 nM each primer, 257 nM FAM (6-carboxy-fluorescein)-labeled PKCβ Taqman probe (5′-FAM-cgctccgtggccttagctgtgc-TAMRA-3′), and 270 nM VIC-labeled β2-microglobulin Taqman probe (5′-VIC-cgctccgtggccttagctgtgc-tamra-3′). Both Taqman probes were conjugated with TAMRA (6-carboxyl-tetramethylrhodamine) as a quencher dye. For an initial

denaturation step, the thermal cycling conditions were set at 94°C for 10 min and 40 cycles at 94°C for 20 s and at 60°C for 1 min.

Chemicals

The chemicals used were *p*-trifluoromethoxy-phenyldrazone, creatine phosphokinase, creatine phosphate, oxytocin (Sigma Chemicals, St Louis, U.S.A.), EDTA, EGTA (Dojindo laboratories, Japan), fura-2/AM (Dojindo, Kumamoto, Japan), phorbol 12,13-dibutylate (PDBu) (Funakosi, Tokyo, Japan), Go6976, Go6983, Go6850, rottlerin (Biomol, Plymouth Meeting, U.S.A.), cremophor EL (Nacalai Tesque, Kyoto, Japan), E64 (protease inhibitor) (Peptide Institute, Osaka, Japan), and ionomycin (Hoechst Japan, Tokyo, Japan). *S. aureus* α-toxin was donated by Dr Iwao Kato (Chiba University, Chiba, Japan). LY333531 was kindly donated by Kissei Pharmaceutical Co. (Matumoto, Nagano, Japan). E64 and ionomycin were dissolved in ethanol. PDBu, fura-2/AM, Go6976, Go6983, Go6850, rottlerin, and LY333531 were dissolved in DMSO. The final concentration of DMSO was $<0.1\%$, which alone had no effect on muscle tension.

Statistics

The results of the experiments were expressed as means ± s.e.m. (n refers to the number of patients). Statistical evaluation of the data was performed using the unpaired Student's t-test for comparisons between pairs of groups and by one- or two-way analysis of variance (ANOVA) followed by either Dunnett's test or the Tukey test for comparisons among more than two groups. A value of $P<0.05$ was taken as significant.

Results

Effects of PDBu on contraction

In the myometrium of nonpregnant women, smooth muscle strips generated spontaneous oscillatory contractions in normal PSS (1.98 cycles per 10 min, $n=13$). In the myometrium of pregnant women, on the other hand, roughly a half of the preparations (eight out of 17 muscles) generated oscillatory contractions (1.33 cycles per 10 min, $n=8$). In the myometrium of either nonpregnant or pregnant women, the addition of PDBu (1 μM) induced a sustained contraction that was maintained for over 2 h (Figure 1a). In nonpregnant myometrium, spontaneous oscillatory contractions were usually superimposed on the sustained contraction. In pregnant myometrium (37–38 weeks), PDBu induced a sustained contraction without oscillatory responses. The contractile force at steady-state level elicited by PDBu was significantly greater in the pregnant myometrium than in the nonpregnant myometrium (Figure 1b) ($n=6$). We also compared the absolute force (expressed as mN mg^{-1} tissue) and phosphorylated MLC in the presence of PDBu (1 μM). The pregnant myometrium exerted greater absolute force than nonpregnant myometrium in the presence of PDBu (Figure 2a) ($n=6$). The antibody used in the present study only recognizes the monophosphorylated MLC at Ser^{19}, that is, with no detectable recognition of nonphosphorylated MLC or diphosphorylated

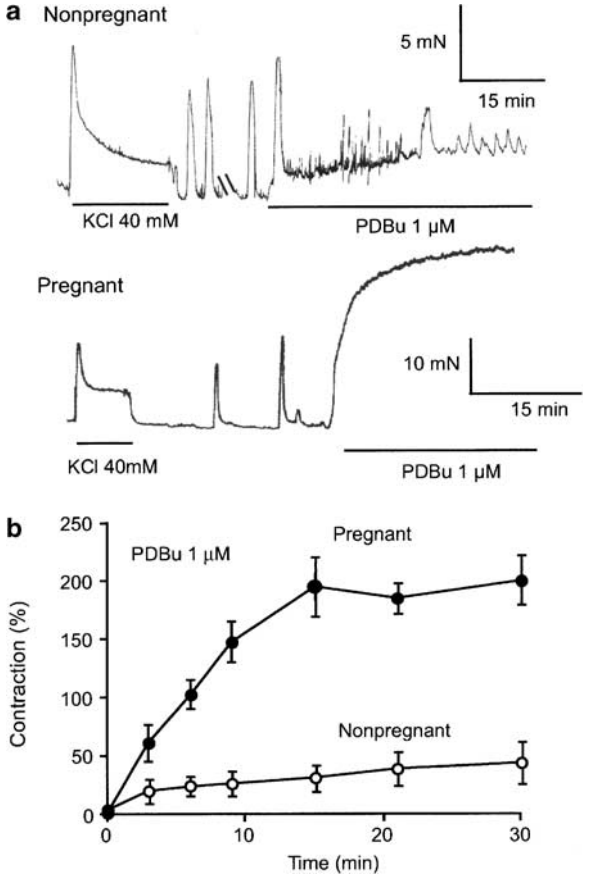

Figure 1 Effects of PDBu on muscle tension in myometria isolated from the pregnant and nonpregnant human uterus. (a) Typical traces of contractile response to PDBu. After the observation of control response to 40 mM K^+, PDBu (1 μM) was added. (b) Comparison of the time courses of contractile activity in pregnant (closed circle) and nonpregnant (open circle) myometria ($n=6$ each). Tension is expressed as percentage of high K^+ (40 mM)-induced contraction.

MLC (Ser^{19} and Thr^{18}) (Sakurada *et al*., 1994). Treatment of the tissue with PDBu (1 μM) for 15 min had no significant effect on MLC phosphorylation in nonpregnant myometrium. However, in pregnant myometrium, the level of MLC phosphorylation increased approximately six-fold (Figure 2b) ($n=6$).

We next examined the effects of various PKC inhibitors on PDBu-induced contractions. The contraction induced by PDBu in the nonpregnant myometrium was not affected by indolocarbasole, Go6976 (1 μM), an inhibitor of conventional PKC (PKCα and PKCβ) (Way *et al*., 2000). In contrast, the contraction induced by PDBu in the pregnant (37–39 weeks) myometrium was greatly inhibited by Go6976 (1 μM). Other inhibitors of conventional PKC, bisindolylmaleimides (1 μM Go6983 and 1 μM Go6850) (data not shown), also strongly inhibited the PDBu-induced contraction in the pregnant myometrium. We also examined the effects of another PKC inhibitor, rottlerin, a selective inhibitor of the novel PKC isoform (PKCδ), and found that at a concentration of 10 μM it did not inhibit the PDBu-induced contraction in either the pregnant (37–39 weeks) or nonpregnant myometrium. These results are summarized in Figure 3 ($n=4$).

We next examined the effect of a selective inhibitor of β PKC isoform, LY333531 (Ishii *et al*., 1996). LY333531 (1 μM)

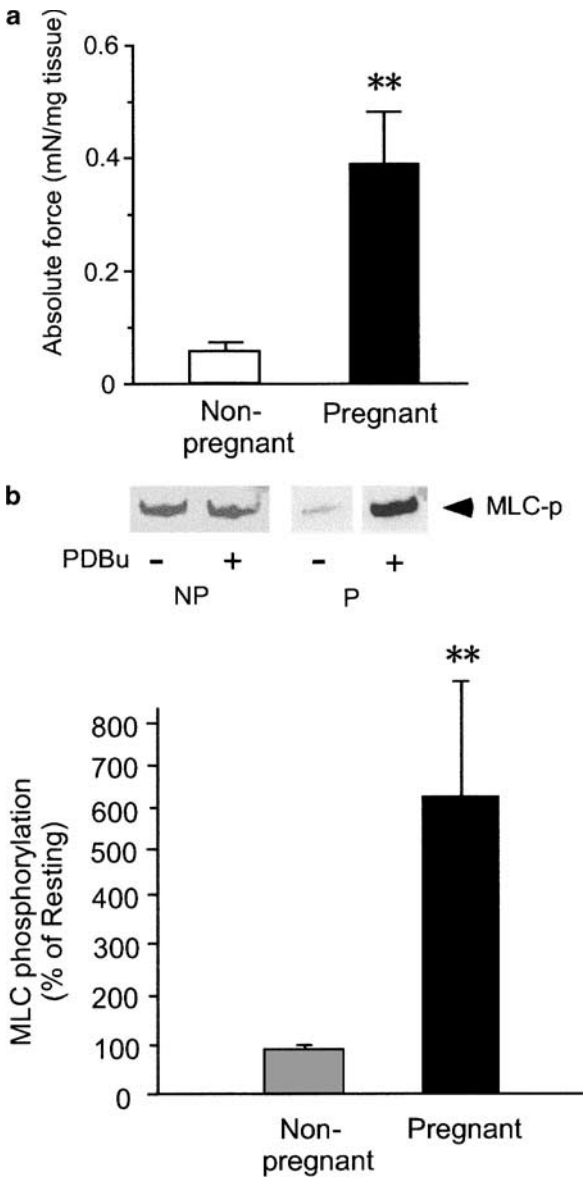

Figure 2 Contractile force and MLC phosphorylation induced by PDBu in pregnant and nonpregnant human uterus. (a) Absolute force of contractions induced by PDBu (1 μM) in nonpregnant and pregnant myometria ($n=6$). Contractile tension at steady-state levels (peak contractile force) was plotted as mN mg^{-1} tissue. (b) 20 kDa MLC phosphorylation induced by (1 μM for 15 min) PDBu in nonpregnant and pregnant (37–38 weeks) myometria ($n=6$). The phosphorylated level of MLC (at serine 19) is expressed as the % ratio of each resting level (mean $\pm$ s.e.m.) (see Methods). $^{**}P<0.01$ vs nonpregnant. Inset indicates the representative Western blotting for MLC phosphorylation.

significantly inhibited the 1 μM PDBu-induced contraction in the pregnant myometrium (37–40 weeks) (Figure 3, right panel) ($n=4$).

Effects of PDBu on the Ca^{2+} sensitivity of contractile elements

To determine the relationship between $[Ca^{2+}]_i$ and contraction in the phorbol ester-stimulated muscles, we measured $[Ca^{2+}]_i$ simultaneously with contractile force. As shown in Figure 4a, in the nonpregnant myometrium ($n=4$), PDBu (1 μM) increased the resting tension with only slightly decreasing the $[Ca^{2+}]_i$ levels. In the pregnant myometrium (37–38 weeks) ($n=4$), PDBu (1 μM) markedly increased the resting tension without changes in the $[Ca^{2+}]_i$ levels (Figure 4b).

To further examine the correlation between $[Ca^{2+}]_i$ and contractile force, we used the α-toxin permeabilized uterine smooth muscles. After the observation of control Ca^{2+} (10 μM)-induced contraction, Ca^{2+} (0.3 and 1 μM) was sequentially added in the absence or presence of 1 μM PDBu (pretreated for 30 min). In the nonpregnant myometrium, PDBu significantly augmented the 0.3 μM Ca^{2+}-induced contraction (Figure 5a). In the pregnant myometrium (37–38 weeks), PDBu significantly augmented the 0.3 and 1 μM Ca^{2+}-induced contractions (Figure 5b). PDBu (1 μM) alone did not change contractile force in the absence of Ca^{2+} ($n=5$).

Effects of PDBu on high K^+-induced increases in $[Ca^{2+}]_i$ and contraction

To determine the role of PKC activation in the stimulated muscles, we next examined the effects of PDBu in the high K^+-depolarized muscles ($n=4$). Figure 6a shows typical traces of the effects of PDBu on $[Ca^{2+}]_i$ and tension in pregnant myometrium (37–38 weeks) stimulated with 40 mM KCl. The PDBu (1 μM), added during the KCl-induced sustained increases in $[Ca^{2+}]_i$ and muscle tension, decreased $[Ca^{2+}]_i$ to the level before the addition of PDBu. Irrespective of the decrease in $[Ca^{2+}]_i$, the contractile force elicited by high K^+ was augmented by PDBu as demonstrated in Figure 6. Although the error bar was large, PDBu increased muscle force in every preparation. After treating the tissue with PDBu, we added verapamil (10 μM). In response to the verapamil treatment, $[Ca^{2+}]_i$ decreased below the resting level and muscle tension slightly decreased.

Western blotting and mRNA

We examined the immunoblotting pattern for monoclonal and polyclonal antibody to various PKC isozymes in human pregnant myometrium. The results indicated that human pregnant myometrium containing conventional PKC (α, β, γ), novel PKC (δ, ε, θ), and atypical PKC (ζ). ι and λ isoforms of atypical PKC could not be visualized under our experimental conditions. The results are essentially similar to those reported by Hurd *et al*. (2000).

As the results from the mechanical study using relatively specific PKC inhibitors suggested that conventional PKC might be involved in the increased contractility of the pregnant myometrium in response to phorbol ester, we examined the changes in mRNA of these isozymes using a semiquantitative RT–PCR method (Figure 7). Among the conventional isoforms of PKC, mRNA levels of α and γ isoforms of PKC did not change after the gestation. In contrast, the mRNA level of β PKC isoform in the pregnant myometrium (37–38 weeks) was significantly greater than that in the nonpregnant myometrium (Figure 7b) ($n=4$). We further carried out quantitative real-time RT-PCR to detect mRNA for PKCβ. As demonstrated in Figure 8, the amount of mRNA for PKCβ significantly increased in the pregnant myometrium ($n=4$).

We also examined the mRNA and protein expression of CPI-17, which is considered to be a substrate for PKC and involved in the smooth muscle contractile elements (Kitazawa

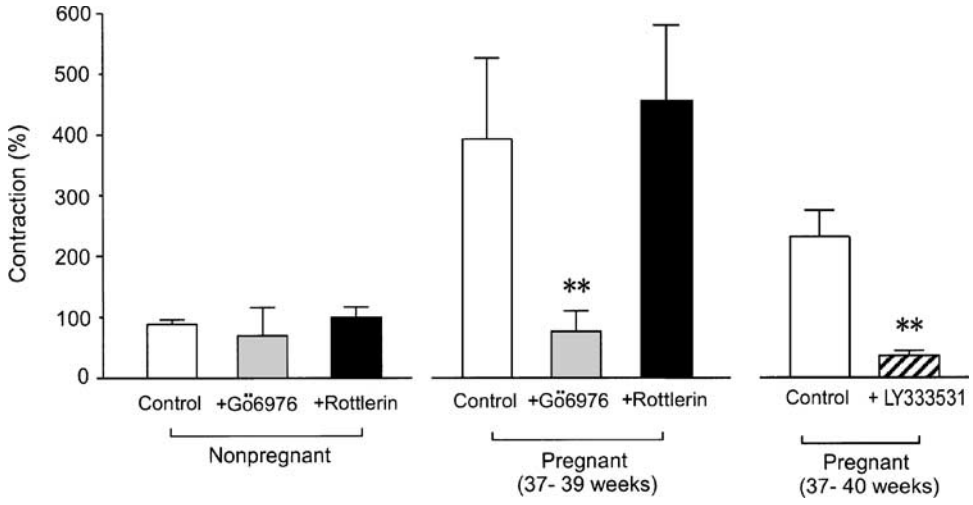

Figure 3 Effect of various PKC inhibitors (Go6976, rottlerin, and LY333531) on PDBu (1 μM)-induced contraction in nonpregnant ($n=4$) and pregnant (37–39 weeks for Go6976 and rottlerin, $n=4$ each; 37–40 weeks for LY333531, $n=4$) human myometrium. After the observation of control response to high K^+ (40 mM), the muscles were contracted by PDBu (1 μM) in the absence or presence of the inhibitors (Go6976 1 μM, rottlerin 10 μM, and LY333531 1 μM). Tension is expressed as percentage of high K^+ (40 mM)-induced contraction. Results are indicated as the mean ± s.e.m. $^*P<0.05$, $^{**}P<0.01$ vs control (PDBu alone).

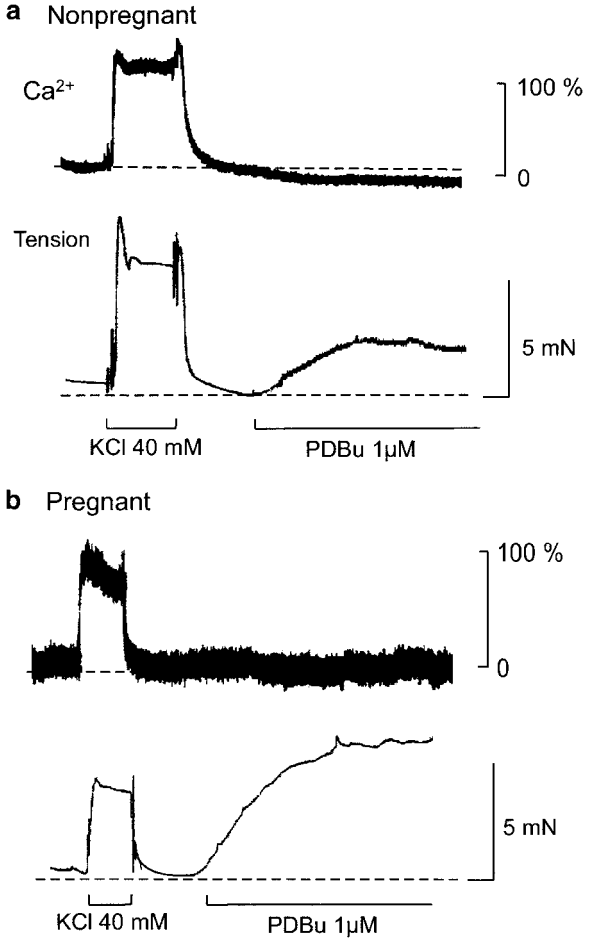

Figure 4 Change in the Ca^{2+} sensitivity of contractile elements in human myometrium as assessed in fura-2-loaded muscles. Effects of PDBu on resting $[Ca^{2+}]_i$ and muscle tension in nonpregnant (a) and pregnant (b) myometria were measured.

et al., 1999; Woodsome *et al*., 2001; Yamawaki *et al*., 2001). Semiquantitative RT–PCR indicated that mRNA for CPI-17 significantly increased in the pregnant myometrium (Figure 9a and b) ($n=9$). Consistently, Western blotting analysis indicated that protein expression of CPI-17 significantly increased in the pregnant myometrium (Figure 10) ($n=4$).

Discussion

It has been reported that PDBu does not induce contractions in rat myometrium (Kim *et al*., 1996). In contrast, the present results demonstrated that PDBu induces a large sustained contraction when added under the resting state in human myometrium. These results suggest the existence of species-dependent differences in the responsiveness to phorbol ester. Furthermore, we observed that the contractile effect of phorbol ester in human myometrium is greater in the pregnant than in the nonpregnant state. The results further demonstrated that PDBu increases the resting tension without increasing $[Ca^{2+}]_i$ (Figure 4). These results lead us to speculate that PKC activation might increase the Ca^{2+} sensitivity of contractile elements in human myometrium, and that this mechanism is augmented in the pregnant state. In various types of smooth muscle, including vascular and tracheal smooth muscles, activation of PKC by phorbol esters is known to increase the Ca^{2+} sensitivity of contractile elements (Kitazawa *et al*., 1989; Ozaki *et al*., 1990; Sato *et al*., 1992). We consistently observed an augmentation of contraction by PDBu at fixed pCa^{2+} in the α-toxin permeabilized human myometrium. In the permeabilized rat myometrium, however, we observed no effects of PDBu on the Ca^{2+}-induced contraction (Kim *et al*., 1996). These results suggest that, distinct from other types of smooth muscle tissues, rat myometrium does not have PKC-mediated Ca^{2+} sensitizing processes to activate contractile elements.

Since phorbol esters directly activate both conventional and novel PKC isozymes, we examined the effect of inhibitors of

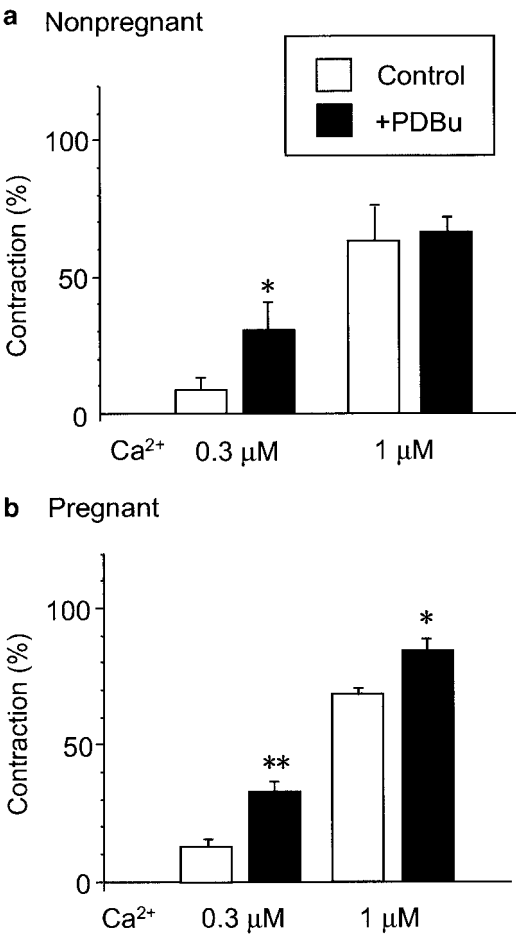

Figure 5 Change in the Ca^{2+} sensitivity of contractile elements in human myometrium as assessed in permeabilized muscles. Ca^{2+}-induced contractions in the absence or presence of PDBu (1 μM) in nonpregnant (a) and pregnant (b) myometria were measured in myometria permeabilized with α-toxin. After the observation of the control response to 10 μM Ca^{2+}, 0.3 and 1 μM Ca^{2+} was sequentially added to induce contractions ($n=5$ each). Contractions were normalized to maximum contraction induced by 10 μM Ca^{2+}. Results are indicated as the mean$\pm$s.e.m. $^{*}P<0.05$, $^{**}P<0.01$ vs control.

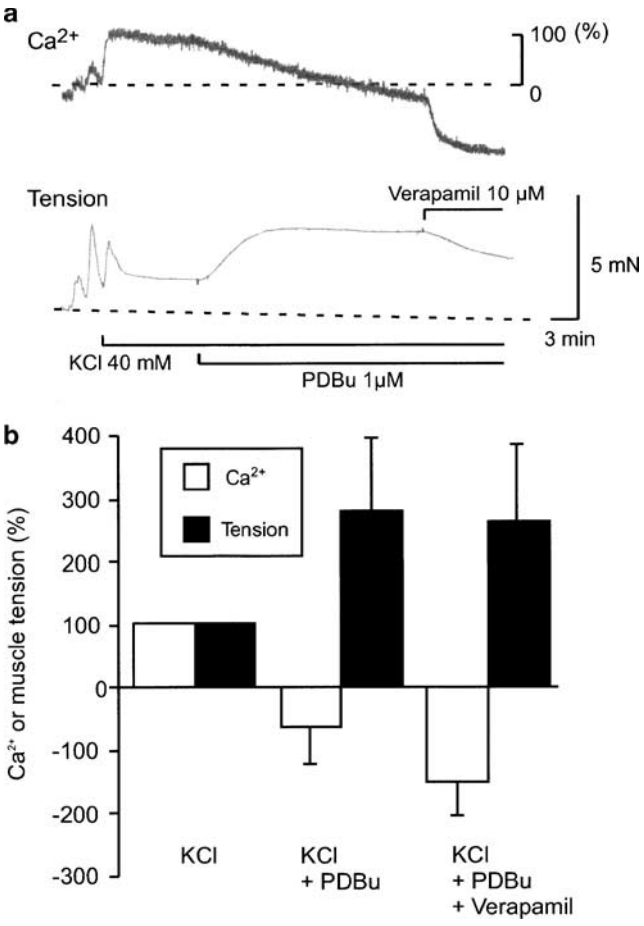

Figure 6 Effects of PDBu on $[Ca^{2+}]_i$ and muscle tension in pregnant human myometrium stimulated with 40 mM KCl. (a) Typical recording of the effects of PDBu (1 μM) on $[Ca^{2+}]_i$ (upper) and contraction (lower). (b) Analyzed data of $[Ca^{2+}]_i$ and force. $[Ca^{2+}]_i$ levels and force at resting were considered as 0% and at high K^+ (40 mM) stimulation (steady state) were considered as 100%. Results are indicated as the mean$\pm$s.e.m. ($n=4$).

these isozymes on PKC-mediated contraction. We first selected Go6976 and rottlerin (Way *et al*., 2000). Rottlerin has been shown to inhibit PKCδ (novel PKC: Ca^{2+}-independent and diacylglycerol-dependent) with some effects over other PKC isoforms. This compound, at a concentration of 10 μM, which induces maximum inhibition of PKCδ (Gschwendt *et al*., 1994), did not inhibit the PDBu-induced contraction either in nonpregnant or pregnant human myometrium. The staurosporin-derived indocarbazole structure has been used to generate a further series of PKC inhibitors, the bisindolylmaleimides. Go6976 (1 μM), which inhibits PKCα and PKCβ (conventional PKC: Ca^{2+}- and diacylglycerol-dependent), strongly inhibited the PDBu-induced sustained contraction. Bisindolylmaleimides, Go6983 and Go6850, both of which preferentially inhibit PKCα, PKCβ, and PKCδ, also induced a marked inhibition of the PDBu-induced contraction in the pregnant human myometrium. These observations suggest that the PKC-mediated contraction, which had been augmented by pregnancy, is attributable to the increased activity of the conventional isoforms of PKC family. LY333531 has been recently shown to inhibit PKCβ with IC_{50} of 4.7–5.9 nM, whereas for other PKC isoenzyme, the IC_{50} was 250 nM or greater (Ishii *et al*., 1996). In the pregnant human myometrium, this compound at 1 μM strongly inhibited the PDBu-induced contraction (Figure 3).

The immunoblotting study indicated that human pregnant myometrium contained various PKC isozymes such as conventional PKC (α, β, γ), novel PKC (δ, ε, θ), and atypical PKCζ. However, the ι and λ isoforms of atypical PKC were not found in the myometrium. Hurd *et al*. (2000) have recently reported, using Western blotting analysis, that PKCβ is absent in nonpregnant myometrium, but is induced during pregnancy. In this study, we confirmed this finding by showing that mRNA for the β isoform was increased in the pregnant myometrium (Figures 8 and 9), leading us to speculate that this PKC isoform may be related to the increased contractility of pregnant myometrium in response to phorbol ester. Although Go6976, an inhibitor of PKCα and PKCβ, inhibited the phorbol ester-induced contraction in pregnant myometrium, the contraction in nonpregnant myometrium was not affected by Go6976. These results suggest that the contractile activity in nonpregnant myometrium is not due to PKCβ isoform, and that myometrial contraction is regulated by multiple PKC isozymes.

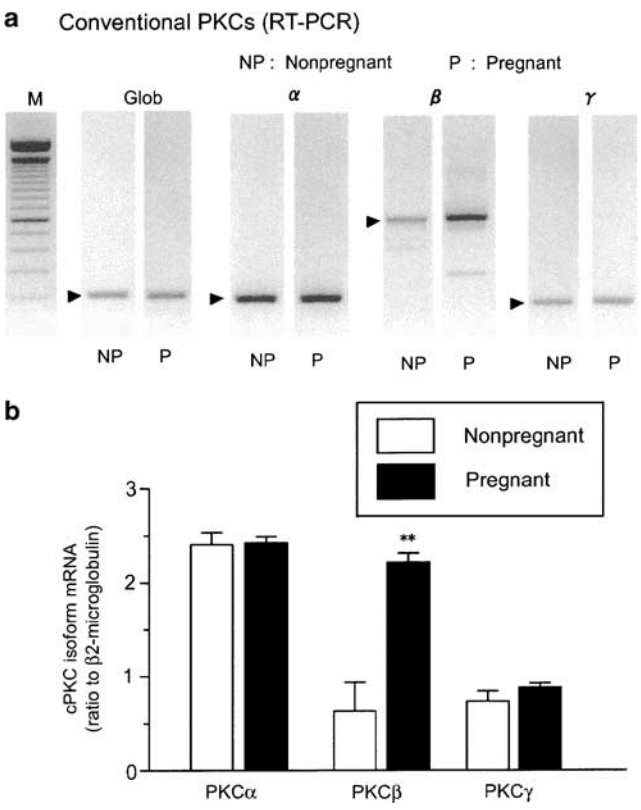

Figure 7 mRNA expressions of conventional PKCs in nonpregnant (NP) and pregnant (P) human myometrial tissues. (a) Typical results of the RT–PCR products at 40 cycles of PCR amplifications. (b) Analytical data for the ratio of β2-microglobulin ($n=4$). $^{**}P<0.01$, pregnant vs nonpregnant tissues.

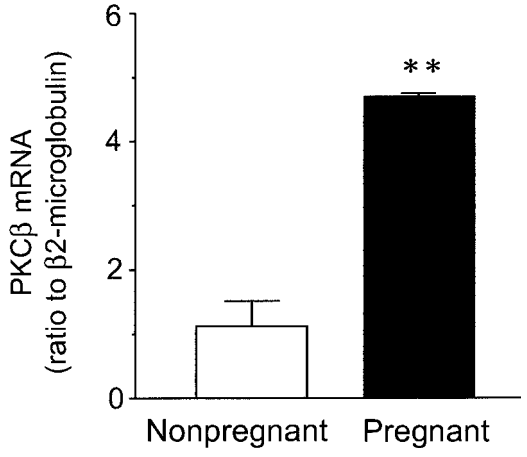

Figure 8 mRNA expressions of PKCβ in nonpregnant and pregnant human myometrial tissues assessed by real-time RT–PCR method. Values are expressed as the ratio of β2-microglobulin ($n=4$). $^{**}P<0.01$, pregnant vs nonpregnant tissues.

MLC phosphorylation is the primary mechanism for activating smooth muscle contraction and occurs principally at Ser[19] of the 20 kDa MLC. In some circumstances, however, Thr[18] phosphorylation may also occur. Using an antibody that selectively recognizes phosphorylated 20kDa MLC at Ser[19], we observed a significant increase in the MLC phosphorylation at Ser[19] in the pregnant myometrium stimulated with 1 μM PDBu (Figure 2b). It is well known that during the stimulation of

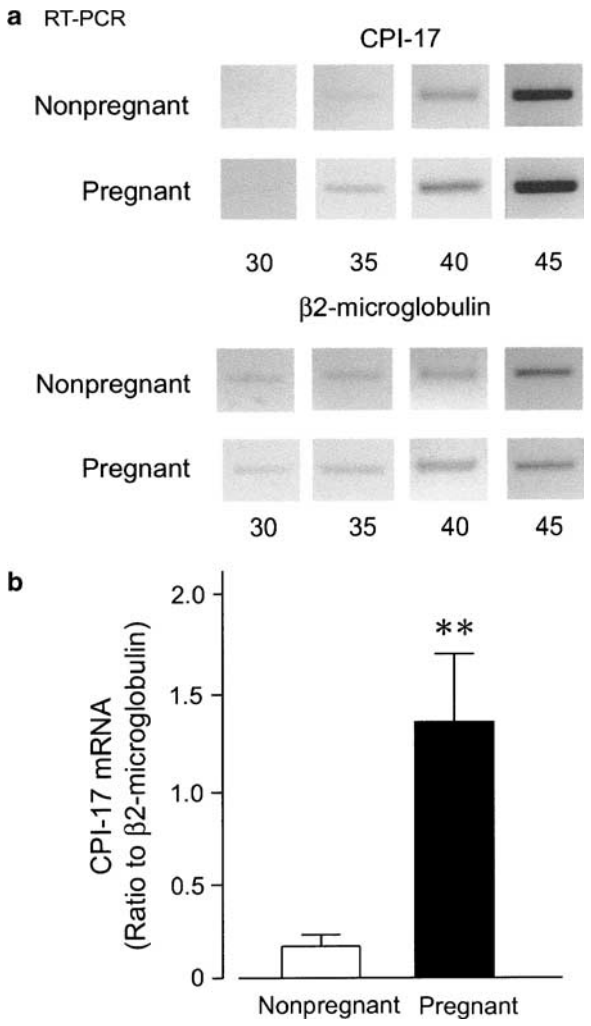

Figure 9 mRNA expressions of CPI-17 in nonpregnant and pregnant human myometrial tissues. (a) Typical results for the RT–PCR products at 30–45 cycles of PCR amplifications. (b) Analytical data for the ratio of β2-microglobulin (at 40 cycles) ($n=9$). $^{**}P<0.01$, pregnant vs nonpregnant tissues.

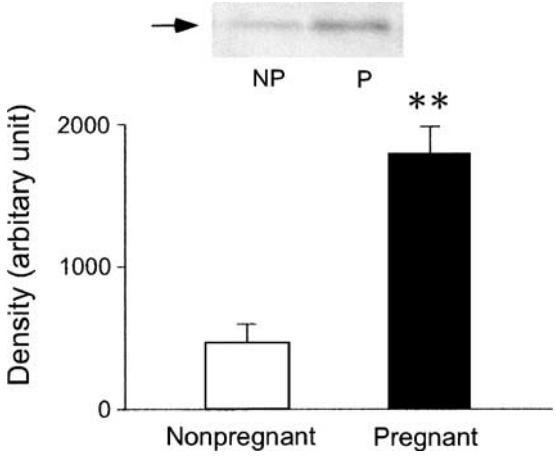

Figure 10 CPI-17 protein expression in nonpregnant and pregnant human myometrial tissues. $^{**}P<0.01$, pregnant vs nonpregnant tissues ($n=4$).

smooth muscle contraction, the Ca^{2+} sensitivity of the contractile element is increased by reducing the myosin phosphatase activity. At least two phosphorylation pathways may be responsible for the increased Ca^{2+} sensitivity: the RhoA/Rho kinase and PKC (Hori & Karaki, 1998). CPI-17, a phosphorylation-dependent inhibitory protein of myosin phosphatase, has been suggested to be the downstream effector of the PKC that may be responsible for the increased contractility (Kitazawa *et al*., 1999; Woodsome *et al*., 2001), as PKC phosphorylates and activates CPI-17. The presence of the human CPI-17 had been detected from cDNA sequences and by Western blots on human aorta (Yamawaki *et al*., 2001). In the present study, we observed that the levels of CPI-17 mRNA and protein significantly increased in the pregnant myometrium. Taken together, the PKC activation by phorbol ester with a high expression of either PKCβ or CPI-17 generated greater contraction in the pregnant myometrium.

Previous reports (Baraban *et al*., 1985; Savineau & Mironneau, 1990; Phillippe, 1994b) have suggested that phorbol ester inhibits the contraction induced by several agonists such as oxytocin and high K^+ in the rat myometrium. In this study, we examined the effects of PDBu on the high K^+-depolarized myometrium, and showed that phorbol esters rather potentiated the contraction of human myometrium. In the rat myometrium, the PDBu-induced inhibition of high K^+-induced contraction was accompanied by a decrease in $[Ca^{2+}]_i$. Notably, in the human myometrium, PDBu increased muscle tension with decreasing $[Ca^{2+}]_i$, showing an apparent dissociation between $[Ca^{2+}]_i$ and force. Therefore, it appears that, unlike other smooth muscles, human myometrium may have a unique positive feedback mechanism for a contractile element coupled to PKC activation. This mechanism might function during labor, during which myometrium is stimulated by several physiological agents. On the other hand, the ability of PKC activation to decrease $[Ca^{2+}]_i$ has been demonstrated in both human (present study) and rat myometrium (Kim *et al*., 1996), which might induce negative feedback regulation following receptor activation. The balance between these two opposite processes may determine the level of contraction.

The uterine smooth muscle undergoes prominent changes during gestation, and increasing evidence indicates the differential expression or activity of functional proteins in human tissue. For example, Perez & Toro (1994) demonstrated that Ca^{2+}-activated large-conductance K^+ channels are modulated differently in pregnant and nonpregnant myometrium. Word *et al*. (1993) demonstrated that the content of contractile elements, such as myosin, actin, caldesmon, and calponin, changes during the course of gestation. The content of cytoskeletal components, such as desmin and vimentin, is also changed after pregnancy (Leoni *et al*., 1990). As PKC targets many proteins in the cell, it is possible that the above-mentioned pathways may also play a role in the PKC-mediated increased contractility of the human pregnant myometrium.

Eude *et al*. (2000) have investigated the role of PKC in contraction of the human myometrium induced by endothelin-1 at the end of pregnancy. The authors indicated that conventional PKC isoforms (α and β), novel PKC isoforms (ε and δ), and an atypical PKC isoform (ζ) were detected in pregnant myometrium. Quantitative immunoblotting further revealed that, of these PKC isoforms, only PKCβ, PKCδ, and PKCζ were translocated to the particulate fraction, and PKCε to the cytoskeletal fraction, after stimulation with endothelin-1. The authors suggested that PKCδ and PKCζ activation mediates endothelin-1-induced contraction, whereas conventional PKC isoforms were not implicated in the human pregnant myometrium. In this study, we have examined if the PKCβ-mediated mechanism is involved in the physiological contractile responses. In the human myometrium, oxytocin stimulated oscillatory contractions, which might be mediated either by the increase in the sensitivity of contractile element. LY333531, a selective inhibitor of PKCβ, inhibited approximately 50% of the oxytocin-induced increase in spontaneous contractions (our unpublished observations). The discrepancy in the role of PKCβ between the contractions induced by oxytocin and entothelin-1 remains unknown at present, and a future study is needed to solve this problem.

Adrenergic β-agonists have been used for the management of preterm labor, as they are effective in suppressing uterine smooth muscle contraction. However, it has been argued that β-agonists are not always beneficial for the treatment of preterm labor because there is a downregulation of β-adrenergic receptors and/or an uncoupling of β-adrenergic receptor to adenylate cyclase *via* Gs in the patients (Litime *et al*., 1989; Dayes & Lye, 1990). The present study in human materials suggests that the inhibition of PKCβ may be a novel therapeutic strategy in the treatment of the preterm labor.

In conclusion, we have found for the first time that PKC activation by phorbol ester, possibly through the PKCβ/CPI-17 pathway, enhances contraction in the pregnant human myometrium with increasing Ca^{2+} sensitivity of contractile elements.

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References

ANWER, K. & SANBORN, B.M. (1989). Changes in intracellular free calcium in isolated myometrial cells: role of extracellular and intracellular calcium and possible involvement of guanine nucleotide-sensitive proteins. *Endocrinology,* **124,** 17–23.

BARABAN, J.M., GOULD, R.J., PEROUTKA, S.J. & SNYDER, S.H. (1985). Phorbol ester effects on neurotransmission: interaction with neurotransmitters and calcium in smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.,* **82,** 604–607.

BREUILLER-FOUCHE, M., TERTRIN-CLARY, C., HELUY, V., FOURNIER, T. & FERRE, F. (1998). Role of protein kinase C in endothelin-1-induced contraction of human myometrium. *Biol. Reprod.,* **59,** 153–159.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.,* **162,** 156–159.

DAYES, B.A. & LYE, S.J. (1990). Characterization of myometrial desensitization to β-adrenergic agonists. *Can. J. Physiol. Pharmacol.,* **68,** 1377–1384.

EUDE, I., PARIS, B., CABROL, D., FERRE, F. & BREUILLER-FOUCHE, M. (2000). Selective protein kinase C isoforms are involved in endothelin-1-induced human uterine contraction at the end of pregnancy. *Biol. Reprod.,* **63,** 1567–1573.

FOMIN, V.P., COX, B.E. & WORD, R.A. (1999). Effect of progesterone on intracellular Ca^{2+} homeostasis in human myometrial smooth muscle cells. *Am. J. Physiol.,* **276,** C379–385.

GSCHWENDT, M., MULLER, H.J., KIELBASSA, K., ZANG, R., KITTSTEIN, W., RINCKE, G. & MARKS, F. (1994). Rottlerin, a novel protein kinase inhibitor. *Biochem. Biophys. Res. Commun.,* **199,** 93–98.

HORI, M. & KARAKI, H. (1998). Regulatory mechanisms of calcium sensitization of contractile elements in smooth muscle. *Life. Sci.,* **62,** 1629–1633.

HURD, W.W., FOMIN, V.P., NATARAJAN, V., BROWN, H.L., BIGSBY, R.M. & SINGH, D.M. (2000). Expression of protein kinase C isozymes in nonpregnant and pregnant human myometrium. *Am. J. Obstet. Gynecol.,* **183,** 1525–1531.

ISHII, H., JIROUSEK, M.R., KOYA, D., TAKAGI, C., XIA, P., CLERMONT, A., BURSELL, S.E., KERN, T.S., BALLAS, L.M., HEATH, W.F., STRAMM, L.E., FEENER, E.P. & KING, G.L. (1996). Amelioration of vascular dysfunctions in diabetic rats by an oral PKC β inhibitor. *Science,* **272,** 728–731.

KARAKI, H. (1989). Ca^{2+} localization and sensitivity in vascular smooth muscle. *Trends. Pharmacol. Sci.,* **10,** 320–325.

KIM, B.K., OZAKI, H., HORI, M. & KARAKI, H. (1996). Increased inhibitory effect of phorbol ester on cytosolic Ca^{2+} level and contraction in rat myometrium after gestation. *Jpn. J. Pharmacol.,* **72,** 111–118.

KIM, B.K., OZAKI, H., LEE, S.M. & KARAKI, H. (1995). Increased sensitivity of rat myometrium to the contractile effect of platelet activating factor before delivery. *Br. J. Pharmacol.,* **115,** 1211–1214.

KITAZAWA, T., KOBAYASHI, S., HORIUTI, K., SOMLYO, A.V. & SOMLYO, A.P. (1989). Receptor-coupled, permeabilized smooth muscle. Role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca^{2+}. *J. Biol. Chem,* **264,** 5339–5342.

KITAZAWA, T., TAKIZAWA, N., IKEBE, M. & ETO, M. (1999). Reconstitution of protein kinase C-induced contractile Ca^{2+} sensitization in triton X-100-demembranated rabbit arterial smooth muscle. *J. Physiol.,* **520,** 139–152.

KUSAKA, M. & SPERELAKIS, N. (1995). Direct block of calcium channels by dioctanoylglycerol in pregnant rat myometrial cells. *Mol. Pharmacol.,* **47,** 842–847.

LEONI, P., CARLI, F. & HALLIDAY, D. (1990). Intermediate filaments in smooth muscle from pregnant and non-pregnant human uterus. *Biochem. J.,* **269,** 31–34.

LITIME, M.H., POINTIS, G., BREUILLER, M., CABROL, D. & FERRE, F. (1989). Disappearance of β-adrenergic response of human myometrial adenylate cyclase at the end of pregnancy. *J. Clin. Endocrinol. Metab.,* **69,** 1–6.

LOIRAND, G., PACAUD, P., MIRONNEAU, C. & MIRONNEAU, J. (1990). GTP-binding proteins mediate noradrenaline effects on calcium and chloride currents in rat portal vein myocytes. *J. Physiol.,* **428,** 517–529.

LONGBOTTOM, E.R., LUCKAS, M.J., KUPITTAYANANT, S., BADRICK, E., SHMIGOL, T. & WRAY, S. (2000). The effects of inhibiting myosin light chain kinase on contraction and calcium signalling in human and rat myometrium. *Pflug. Arch.,* **440,** 315–321.

MARC, S., LEIBER, D. & HARBON, S. (1986). Carbachol and oxytocin stimulate the generation of inositol phosphates in the guinea pig myometrium. *FEBS Lett.,* **201,** 9–14.

MORGAN, J.P. & MORGAN, K.G. (1984). Stimulus-specific patterns of intracellular calcium levels in smooth muscle of ferret portal vein. *J. Physiol.,* **351,** 155–167.

MORRISON, J.J., DEARN, S.R., SMITH, S.K. & AHMED, A. (1996). Activation of protein kinase C is required for oxytocin-induced contractility in human pregnant myometrium. *Hum. Reprod.,* **11,** 2285–2290.

NISHIMURA, J., KOLBER, M. & VAN BREEMEN, C. (1988). Norepinephrine and GTP-γ-S increase myofilament Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle. *Biochem. Biophys. Res. Commun.,* **157,** 677–683.

NISHIZUKA, Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.,* **9,** 484–496.

OZAKI, H., KWON, S.C., TAJIMI, M. & KARAKI, H. (1990). Changes in cytosolic Ca^{2+} and contraction induced by various stimulants and relaxants in canine tracheal smooth muscle. *Pflug. Arch.,* **416,** 351–359.

OZAKI, H., SATO, K., SATOH, T. & KARAKI, H. (1987). Simultaneous recordings of calcium signals and mechanical activity using fluorescent dye fura 2 in isolated strips of vascular smooth muscle. *Jpn. J. Pharmacol.,* **45,** 429–433.

OZAKI, H., ZHANG, L., BUXTON, I.L., SANDERS, K.M. & PUBLICOVER, N.G. (1992). Negative-feedback regulation of excitation–contraction coupling in gastric smooth muscle. *Am. J. Physiol.,* **263,** C1160–1171.

PEREZ, G. & TORO, L. (1994). Differential modulation of large-conductance K_{Ca} channels by PKA in pregnant and nonpregnant myometrium. *Am. J. Physiol.,* **266,** C1459–1463.

PHILLIPPE, M. (1994a). Mechanisms underlying phasic contractions of pregnant rat myometrium stimulated with aluminum fluoride. *Am. J. Obstet. Gynecol.,* **170,** 981–988.

PHILLIPPE, M. (1994b). Protein kinase C, an inhibitor of oxytocin-stimulated phasic myometrial contractions. *Biol. Reprod.,* **50,** 855–859.

SAKATA, K. & KARAKI, H. (1992). Effects of endothelin on cytosolic Ca^{2+} level and mechanical activity in rat uterine smooth muscle. *Eur. J. Pharmacol.,* **221,** 9–15.

SAKATA, K., OZAKI, H., KWON, S.C. & KARAKI, H. (1989). Effects of endothelin on the mechanical activity and cytosolic calcium level of various types of smooth muscle. *Br. J. Pharmacol.,* **98,** 483–492.

SAKURADA, K., IKUHARA, T., SETO, M. & SASAKI, Y. (1994). An antibody for phosphorylated myosin light chain of smooth muscle: application to a biochemical study. *J. Biochem. (Tokyo),* **115,** 18–21.

SATO, K., HORI, M., OZAKI, H., TAKANO-OHMURO, H., TSUCHIYA, T., SUGI, H. & KARAKI, H. (1992). Myosin phosphorylation-independent contraction induced by phorbol ester in vascular smooth muscle. *J. Pharmacol. Exp. Ther.,* **261,** 497–505.

SATO, K., OZAKI, H. & KARAKI, H. (1988). Changes in cytosolic calcium level in vascular smooth muscle strip measured simultaneously with contraction using fluorescent calcium indicator fura 2. *J. Pharmacol. Exp. Ther.,* **246,** 294–300.

SAVINEAU, J.P. & MIRONNEAU, J. (1990). An analysis of the action of phorbol 12, 13-dibutyrate on mechanical activity in rat uterine smooth muscle. *J. Pharmacol. Exp. Ther.,* **255,** 133–139.

SCHIEMANN, W.P., DOGGWILER, K.O. & BUXTON, I.L. (1991). Action of adenosine in estrogen-primed nonpregnant guinea pig myometrium: characterization of the smooth muscle receptor and coupling to phosphoinositide metabolism. *J. Pharmacol. Exp. Ther.,* **258,** 429–437.

SCHREY, M.P., CORNFORD, P.A., READ, A.M. & STEER, P.J. (1988). A role for phosphoinositide hydrolysis in human uterine smooth muscle during parturition. *Am. J. Obstet. Gynecol.,* **159,** 964–970.

SOMLYO, A.P. & SOMLYO, A.V. (1994). Signal transduction and regulation in smooth muscle. *Nature,* **372,** 231–236.

SZAL, S.E., REPKE, J.T., SEELY, E.W., GRAVES, S.W., PARKER, C.A. & MORGAN, K.G. (1994). $Ca^{2+}]_i$ signaling in pregnant human myometrium. *Am. J. Physiol.,* **267,** E77–E87.

WAY, K.J., CHOU, E. & KING, G.L. (2000). Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol. Sci.,* **21,** 181–187.

WEBB, B.L., HIRST, S.J. & GIEMBYCZ, M.A. (2000). Protein kinase C isoenzymes: a review of their structure, regulation and role in regulating airways smooth muscle tone and mitogenesis. *Br. J. Pharmacol.,* **130,** 1433–1452.

WOODSOME, T.P., ETO, M., EVERETT, A., BRAUTIGAN, D.L. & KITAZAWA, T. (2001). Expression of CPI-17 and myosin phosphatase correlates with Ca^{2+} sensitivity of protein kinase C-induced contraction in rabbit smooth muscle. *J. Physiol.,* **535,** 553–564.

WORD, R.A., STULL, J.T., CASEY, M.L. & KAMM, K.E. (1993). Contractile elements and myosin light chain phosphorylation in myometrial tissue from nonpregnant and pregnant women. *J. Clin. Invest.,* **92,** 29–37.

WORD, R.A., TANG, D.C. & KAMM, K.E. (1994). Activation properties of myosin light chain kinase during contraction/relaxation cycles of tonic and phasic smooth muscles. *J. Biol. Chem.,* **269,** 21596–21602.

WRAY, S. (1993). Uterine contraction and physiological mechanisms of modulation. *Am. J. Physiol.,* **264,** C1–18.

YAMAWAKI, K., ITO, M., MACHIDA, H., MORIKI, N., OKAMOTO, R., ISAKA, N., SHIMPO, H., KOHDA, A., OKUMURA, K., HARTSHORNE, D.J. & NAKANO, T. (2001). Identification of human CPI-17, an inhibitory phosphoprotein for myosin phosphatase. *Biochem. Biophys. Res. Commun.,* **285,** 1040–1045.

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