PKCδ Mediates Testosterone-induced Increases in Coronary Smooth Muscle Ca$_{\text{v}}$1.2*

Received for publication, August 19, 2005, and in revised form, October 20, 2005. Published, JBC Papers in Press, October 21, 2005, DOI 10.1074/jbc.M509147200

Kamala K. Maddali, Donna H. Korzick, Darla L. Tharp, and Douglas K. Bowles

From the Department of Biomedical Sciences, Dalton Cardiovascular Research Center, National Center for Gender Physiology, University of Missouri, Columbia, Missouri 65211 and Department of Kinesiology and Program in Physiology, The Pennsylvania State University, University Park, Pennsylvania 16802

Sex hormones have emerged as important modulators of cardiovascular physiology and pathophysiology. Our previous studies demonstrated that testosterone increases expression and activity of L-type, voltage-gated calcium channels (Ca$_{\text{v}}$1.2) in coronary arteries of males. The purpose of the present study was to determine whether testosterone (T) alters coronary protein kinase C δ (PKCδ) expression and whether PKCδ plays a role in coronary Ca$_{\text{v}}$1.2 expression. For in vitro studies, porcine right coronary arteries (RCA) and post-confluent (passages 3–6) 5-day, serum-restricted expression. For L-type, voltage-gated calcium channels (Cav1.2) in coronary arteries demonstrated that testosterone increases expression and activity of vascular physiology and pathophysiology. Our previous studies demonstrated that testosterone increases expression and activity of L-type, voltage-gated calcium channels (Ca$_{\text{v}}$1.2) in coronary arteries of males. The purpose of the present study was to determine whether testosterone (T) alters coronary protein kinase C δ (PKCδ) expression and whether PKCδ plays a role in coronary Ca$_{\text{v}}$1.2 expression.

In vitro T and dihydrotestosterone caused a 2–3-fold increase in PKCδ protein levels, ~1.5–2-fold increase in PKCδ kinase activity, and localization of PKCδ toward the plasma membrane and nuclear envelope. PKCδ protein levels were higher in coronary arteries of intact males compared with intact females. Elimination of endogenous testosterone by castration reduced RCA PKCδ protein levels, an effect partially (~45%) reversed by exogenous T (castrated males with T replacement). In CSMC, PKC inhibition with either the general PKC inhibitor, chelerythrine, or the putative PKCδ inhibitor, rottlerin, completely inhibited the T-mediated increase in coronary Ca$_{\text{v}}$1.2 protein levels. Conversely, Go6976, a conventional PKC isoform inhibitor, failed to inhibit T-induced increases in coronary Ca$_{\text{v}}$1.2 protein levels. PKCδ short interference RNA completely blocked T-induced increases in Ca$_{\text{v}}$1.2 protein levels in CSMC. These results demonstrate for the first time that 1) endogenous T is a primary modulator of coronary PKCδ protein and activity in males and 2) T increases Ca$_{\text{v}}$1.2 protein expression in a PKCδ-dependent manner.

Coronary heart disease is a major cause of global mortality and represents an underlying cause for most heart attacks and sudden death (1). Men 30–50 years of age have an increased incidence of coronary heart disease compared with women of similar age (2–4), a sex difference that led many to the conclusion that testosterone increases the risk of coronary heart disease in men. However, recent clinical studies have failed to support a detrimental effect of testosterone on coronary heart disease (5–7). On the contrary, low testosterone concentrations in men are associated with a higher risk of atherosclerosis (7). Men with low testosterone levels are often more obese, hypertensive, and have increased blood glucose and serum cholesterol levels and increased carotid artery atherosclerosis with diabetes (5, 7). Additionally, Dunajaska et al. (6) have shown that low levels of total testosterone, testosterone/estriadiol ratio, and free androgen index are associated with coronary artery disease in men. These findings underscore the need to fully understand the effects of testosterone on coronary vascular wall biology.

Sex hormones exert multiple and diverse effects on the vascular wall. Both endothelial and vascular smooth muscle cells express estrogen (8) and androgen receptors (9, 10). We recently provided the first evidence for an increased L-type voltage-gated calcium channel (Ca$_{\text{v}}$1.2) current in coronary artery smooth muscle of male swine compared with females (11). Subsequently, we demonstrated that coronary Ca$_{\text{v}}$1.2 expression and activity are stimulated by endogenous testosterone in males (12). In vitro, both testosterone and non-aromatizable androgen, dihydrotestosterone (DHT), increased Ca$_{\text{v}}$1.2 protein levels (12). Endogenous testosterone increases Ca$_{\text{v}}$1.2 expression in porcine coronary smooth muscle cells. However, the mechanisms by which androgens elevate Ca$_{\text{v}}$1.2 protein levels in males are poorly understood.

Many aspects of vascular smooth muscle biology, including contraction, differentiation, proliferation, apoptosis, and myogenic responses, share common signaling pathways involving the activation of protein kinase C (PKC) (13, 14). For example, acute activation of PKC with phorbol esters increases Ca$^{2+}$ influx in vascular smooth muscle, purportedly via PKC-dependent activation of voltage-gated Ca$^{2+}$ channels (15, 16). Kanashiro et al. (17) showed an increased expression and activity of PKCa, -δ, and -γ in aortas from intact males and ovariectomized females compared with intact females, revealing an isoform-specific effect of sex hormones, specifically estrogen, to PKC expression and activity. We previously reported a 4-fold greater expression of PKCδ protein levels in male coronary arteries compared with females, a sex difference that was eliminated by castration of males (18), suggesting increased PKCδ levels in males might be driven by male sex hormones, e.g. testosterone.

Despite evidence for an important regulatory role of PKC in vasoreactivity and vascular disease progression, to our knowledge no information exists regarding androgen effects on PKCδ expression and its role in coronary Ca$_{\text{v}}$1.2 protein expression. In the present study, we therefore compared expression, activity, and subcellular distribution of PKCδ in coronary smooth muscle following testosterone (T) stimulation and, in addition, tested the hypothesis that testosterone-induced increases in Ca$_{\text{v}}$1.2 were PKCδ dependent. The results demonstrate that androgens increase both

---

* This study was supported by NHLBI, National Institutes of Health Grant HL071574 and by the National Aeronautics and Space Administration. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: E102 Veterinary Medicine, University of Missouri, Columbia, MO 65211. Tel.: 573-882-7193; Fax: 573-884-6890; E-mail: BowlesD@missouri.edu.

2 The abbreviations used are: DHT, dihydrotestosterone; PKC, protein kinase C; T, testosterone; CM, castrated male; RCA, right coronary artery; SFM, serum-free medium; CSMC, coronary smooth muscle cell; siRNA, short interfering RNA.
PKCδ activity and expression, which serve as a mandatory intermediate for testosterone-induced increases in Ca_{1.2} protein levels.

**MATERIALS AND METHODS**

**Animals**—Sexually mature male and female Yucatan swine were obtained from the breeder (Sinclair Research Farm; Columbia, MO) and housed in pens at the College of Veterinary Medicine. Animal protocols were approved by the University of Missouri Animal Care and Use Committee in accordance with the “Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training” as described previously (12).

**Castration and Hormone Replacement**—Data for PKCδ in swine with altered hormone status were obtained from coronary arteries of swine utilized in a previous study (12). As described previously (12), castration (orchietomy) and hormone replacement were performed by the Swine Hormone Core within the National Center for Gender Physiology. One week after arrival, sexually mature animals (6–7 months of age) were castrated with aseptic techniques under sedation with xylazine (15 mg/kg im) and ketamine (2.5 mg/kg intramuscular) and maintained under anesthesia with 1.5–2.0% isoflurane. Males were castrated (CM) via incision made through the scrotum over each testicle (intact males; IM) and subsequently randomized to receive testosterone replacement (CMT; 10 mg/day; Androgel, Solvay Pharmaceuticals) or vehicle. Testosterone replacement occurred at the time of castration to avoid disruption of hormonal influence. Females remained gonadally intact. Animals were euthanized for study 5–6 weeks after surgery.

**Isolation of Coronary Arteries**—Miniature swine were anesthetized with ketamine (35 mg/kg), rompun (2.25 mg/kg), and pentothal sodium (10 mg/kg) followed by administration of heparin (1000 units/kg). Swine were euthanized by removal of the heart, and the heart was placed in 4 °C PSS. The right coronary artery (RCA) was isolated, cleaned of fat and connective tissue, and placed in low Ca²⁺ physiological saline solution containing 20 mM HEPES at 4 °C.

**Coronary Artery Culture and Treatment**—An intact coronary artery tissue culture model was used to study sex hormone effects on coronary artery smooth muscle in vitro as modified from Hill et al. (19) and Maddali et al. (20). RCA (intact vessels) were cut into 1-cm rings and incubated for 18 h in SFM at 37 °C in a humidified chamber.

**Immunocytofluorescence**—Freshly dispersed coronary smooth muscle cells were obtained enzymatically from RCA segments as previously described (20). Cells were fixed on polyllysine-coated coverslips (BD Biosciences) in 4% paraformaldehyde, permeabilized in phosphate-buffered saline plus 0.1% Triton X-100, and incubated in phosphate-buffered saline plus 5% bovine serum albumin and 5% goat serum for 30 min. Cells were washed and incubated overnight at 4 °C in primary rabbit polyclonal antibodies for PKCδ (1:1000) and tagged with goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR). Nuclei were visualized with the nuclear dye, propidium iodide (5 × 10⁻⁷ M, 10 min; Molecular Probes). Cells were obtained using a ×60 water immersion objective (1.2 NA) on an Olympus IX-70 (Tokyo, Japan) inverted microscope coupled with a Bio-Rad Radiance-2000 confocal system. Optical sections (10–15) were obtained at 0.7-μm intervals along the z-axis. Specificity of secondary antibody was verified in all experiments by addition of secondary antibody in the absence of primary antibody, which showed no fluorescent signal (not shown). Cells prepared from five animals were dispersed onto eight slides. Approximately 40–50 cells were imaged for PKCδ isoform, and representative images were presented.

**PKCδ siRNA Protocol**—Transfection was performed according to the manufacturer’s protocol (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, 5 μl of Lipofectamine 2000 (Invitrogen) and 20 pm PKCδ siRNA or control siRNA pool (Santa Cruz Biotechnology) were diluted in 250 μl of serum-free phenol red-free Dulbecco’s modified Eagle’s medium and incubated at room temperature for not more than 5 min. The diluted siRNA pools were then mixed with the diluted Lipofectamine 2000 and incubated at room temperature for 20 min. For transfection, post-confluent cells were subcultured at 90% confluence and serum starved for 2 days. Cells were then incubated at 37 °C for 6 h with siRNA-Lipofectamine 2000 complexes. After the transfection period, the medium was removed, the cells were washed twice with SFM, and 2 ml of fresh SFM was added. The cultures were then allowed to recover for 48 h with SFM changes every 24 h. At the end of the 48-h recovery period, the cells in 1–2 wells from each siRNA group (control and PKCδ) were harvested and cell numbers were evaluated. The remaining wells in each group were either treated with ethanol (vehicle controls) or T (100 nM). The total treatment time was 18 h. The cells in each group were collected, pelleted, and used for immunoblot analysis. All the siRNA experiments were conducted in serum-free medium containing no antibiotics.

**Materials**—The following were purchased from Sigma: phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, glycerol, and 0.25% Triton X-100. Homogenates were immunoprecipitated with an antibody against PKCδ using protein G-agarose (Amersham Biosciences). The immunocomplexes were washed three times with phosphorylation lysis buffer and two times with kinase buffer (25 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.5 mM EGTA, 1 mM dithiothreitol, 20 mg of phosphatidylserine, and 20 mM ATP, 25 mM MgCl₂, 25 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate) and were resuspended in 30 μl of kinase buffer containing 5 mg of histone H1, and then 20–30 μCi of [γ⁻³²P]ATP was added. The reaction was incubated for 15–30 min at room temperature and was terminated by the addition of SDS-sample buffer. Proteins were analyzed by SDS-PAGE, and the phosphorylated form of histone H1 was detected by autoradiography.
Testosterone, Ca

,

1.2, and Coronary PKC\(\delta\)

RPMI 1640 medium, phosphate-buffered saline, goat serum, and rabbit serum were obtained from Invitrogen. Antibodies to PKC isoforms were obtained from Santa Cruz Biotechnology. Antibody to Ca

,1.2 was purchased from Alomone Labs (Jerusalem, Israel). Molecular mass markers and Tris-HCl gels were purchased from Bio-Rad. Horseradish peroxidase-conjugated goat anti-rabbit IgG and ECL Western blotting detection reagents were obtained from Amersham Biosciences.

Statistical Analysis—All values are expressed as mean \(\pm\) S.E. Comparisons among treatment groups were made using analysis of variance with Bonferroni post hoc analyses when indicated. A \(p\) value \(\leq 0.05\) was set as the criterion for significance in all comparisons.

RESULTS

To determine whether the sex differences in PKC\(\delta\) expression were driven by testosterone, we examined the influence of sex on PKC\(\delta\) protein in an in vitro model. As we have previously demonstrated (18), males were found to have greater PKC\(\delta\) compared with intact females (Fig. 1). Castration markedly reduced PKC\(\delta\) protein levels in males, whereas testosterone replacement partially (~45%) prevented the loss of protein due to castration (Fig. 1). Thus, endogenous testosterone increases coronary PKC\(\delta\) protein and likely contributes to greater PKC\(\delta\) protein levels in coronary arteries of males.

To further investigate the mechanism of testosterone stimulation of PKC\(\delta\) protein expression (Fig. 1), we determined the effects of testosterone on PKC\(\delta\) protein levels in an in vitro cell culture model and organ culture model (intact RCA). For this, PKC\(\delta\) expression was determined in homogenates obtained from intact RCA and post-confluent, 5-day serum-restricted coronary smooth muscle cells incubated with testosterone or dihydrotestosterone. In intact vessels, both testosterone and dihydrotestosterone produced similar, concentration-dependent increases in PKC\(\delta\) protein with a maximal response of ~2.5- and ~3.5-fold, respectively, at 100 nM (Fig. 2, A and C). We observed a similar response in CSMC (Fig. 2, B and D), whereby PKC\(\delta\) protein levels increased ~2.3- and ~3.6-fold following testosterone and dihydrotestosterone treatment, respectively. Together with intact vessel data, these results confirm the up-regulation of PKC\(\delta\) protein levels by testosterone. In vivo, testosterone can be converted to either estrogen or dihydrotestosterone by aromatase or 5-α reductase, respectively. Dihydrotestosterone is a non-aromatizable androgen that acts through binding to the androgen receptor. The similar effects of testosterone and DHT on PKC\(\delta\) protein levels provide strong evidence that testosterone conversion to estrogen by aromatization is not necessary for these observed effects of testosterone on PKC\(\delta\).

To determine whether testosterone affects PKC\(\delta\) kinase activity,
PKCδ protein was immunoprecipitated from homogenates of intact vessels and cultured CSMC treated with testosterone (10 or 100 nM) or DHT (10 or 100 nM). Testosterone treatment significantly elevated PKCδ kinase levels in both intact vessels and primary CSMC in a concentration-dependent manner (Fig. 3, A and B). Densitometric analysis indicated ~1.5- and ~3-fold increases in total PKCδ activity with 10 and 100 nM testosterone, respectively. DHT also increased PKCδ kinase activity in a concentration-dependent manner (~5- and ~7-fold for 10 and 100 nM, respectively) in both intact vessels and CSMC (Fig. 3, A and B). These data provide evidence that a non-aromatizable androgen, DHT, mimics the effects of testosterone on PKCδ kinase activity.

To evaluate the subcellular distribution of PKCδ in response to testosterone, CSMC were dispersed from testosterone-treated RCA and subcellular distribution examined by confocal immunocytochemistry. In untreated cells, PKCδ was diffusely distributed throughout the cytosol (Fig. 3C, left). Conversely, cells treated with 100 nM testosterone showed a localization of PKCδ toward plasma membrane and a perinuclear, reticular pattern that extended into the central cytosol (Fig. 3C, right). These data demonstrate a specific subcellular distribution of PKCδ in response to testosterone, suggesting a role for PKCδ in membrane and nuclear signaling.

We have previously demonstrated that testosterone drives Ca_{1,2} mRNA and protein expression in coronary smooth muscle (12). The finding in the present study that testosterone also increases coronary smooth muscle PKCδ protein levels is consistent with previous sex differences in coronary PKCδ protein levels (18) and led us to determine the contribution of PKCδ in testosterone-mediated effects on Ca_{1,2} protein expression. RCA segments and primary CSMC were pretreated with chelerythrine (10 μM), a general inhibitor of PKC, Go6976 (5 μM), a conventional PKC isoform inhibitor, or rottlerin (5 μM), a putative PKCδ-selective inhibitor, and incubated for 18 h in the presence/absence of testosterone (100 nM). Both chelerythrine and rottlerin inhibited testosterone-induced Ca_{1,2} protein levels (Fig. 4). In contrast, Go6976 had no effect on T-induced Ca_{1,2} protein levels. These data demonstrate that testosterone stimulation of Ca_{1,2} protein expression in coronary smooth muscle is mediated by PKCδ and/or novel PKC isoforms with no apparent contribution from conventional PKC isoforms.

To further test the hypothesis that PKCδ is the specific isoform involved in testosterone up-regulation of Ca_{1,2}, suppression of PKCδ protein was accomplished using siRNA (Fig. 5). Successful RNA silencing was demonstrated by knockdown of PKCδ protein by ~90% (Fig. 5). To demonstrate specificity, siRNA-treated CSMC were probed for additional PKC isoforms, i.e. PKCa, -ε, and -ζ (Fig. 5). PKCδ siRNA essentially abolished PKCδ protein levels without altering the expression of PKCa, -ε, and -ζ in CSMC, providing evidence for specific down-regulation of PKCδ. Importantly, PKCδ siRNA completely blocked the stimulatory effect of testosterone on Ca_{1,2} protein expression (Fig. 6). These data demonstrate that testosterone increases Ca_{1,2} protein expression through a PKCδ-dependent mechanism.

**DISCUSSION**

The present study provides several novel insights into testosterone regulation of coronary arteries, including that 1) stimulation of coronary PKCδ by endogenous testosterone in vivo, 2) stimulation of coronary smooth muscle PKCδ expression and activity by both testosterone and dihydrotestosterone in vitro, and 3) testosterone-induced increases in Ca_{1,2} expression in coronary smooth muscle are dependent upon increases in PKCδ. Specifically, testosterone increased PKCδ protein levels and activity and altered the subcellular localization of PKCδ. In addition, these studies provide a mechanism for our previous finding that endogenous testosterone increases in Ca_{1,2} protein levels, mRNA expression, and activity in male...
Testosterone, Ca\textsubscript{v}1.2, and Coronary PKC\textsubscript{δ}

FIGURE 4. Testosterone increases coronary Ca\textsubscript{v}1.2 protein via PKC. Densitometric analysis of the inhibitory effects of Go6976 (5 \(\mu\)M), chelerythrine chloride (10 \(\mu\)M), and rottlerin (5 \(\mu\)M) on testosterone-induced Ca\textsubscript{v}1.2 protein expression (n = 5). Each sample was normalized to the average control value obtained from the same blot. Top, representative immunoblot showing CSMC whole cell homogenates probed with anti-Ca\textsubscript{v}1.2 antibody from control and testosterone (T)-treated cells in the presence and absence of corresponding PKC inhibitors. CSMC were incubated for 18 h in the absence or presence of testosterone with and without Go6976 (conventional PKC inhibitor), chelerythrine chloride (a generalized PKC inhibitor), and rottlerin (putative PKC\textsubscript{δ} inhibitor). Chelelerythrine and rottlerin inhibited testosterone up-regulation of Ca\textsubscript{v}1.2 protein. In contrast, Go6976 failed to inhibit testosterone up-regulation of Ca\textsubscript{v}1.2 protein. Ca\textsubscript{v}1.2-positive band appeared at \(-220\) kDa. Values are mean ± S.E. \(p < 0.05\) versus all controls and † versus T in the absence of PKC inhibitors.

FIGURE 5. Specific down-regulation of coronary PKC\textsubscript{δ} protein siRNA. Representative immunoblots showing PKC\textsubscript{δ} siRNA-treated and untreated CSMC whole cell homogenates probed with anti-PKC\textsubscript{δ}, -α, -ε, and -ζ antibodies. For the evaluation of protein loading, the blots were stripped and reprobed for β-actin. PKC\textsubscript{δ} siRNA successfully knocked down PKC\textsubscript{δ} protein levels without altering the expression of PKC α, ε, and -ζ in CSMC, providing evidence for specific down-regulation of PKC\textsubscript{δ} protein levels. Autoradiograms are representative of four independent experiments for each isoform.

Coronary arteries (12). Furthermore, these findings demonstrate that testosterone, within the physiological range of total serum testosterone in humans (21), is a selective and potent regulator of PKC\textsubscript{δ} protein levels in coronary artery smooth muscle.

Recent studies have concluded that sex differences in PKC contribute to differences in vascular smooth muscle reactivity (17, 18). In the rat aorta, estrogen has been identified as the primary mediator of sex differences in vascular reactivity and PKCα, -δ, and -ζ expression, with no effect of testosterone (17, 22). However, in our porcine model, castration reduced, and testosterone replacement abrogated, coronary PKC\textsubscript{δ} protein levels. The efficacy of castration and testosterone replacement in these animals has been previously published (12); thus coronary PKC\textsubscript{δ} levels directly correlate with endogenous testosterone levels. It is of interest to note that testosterone replacement did not completely prevent the loss of PKC\textsubscript{δ} despite maintenance of total serum testosterone levels similar to those in intact controls (12). This could be due to our inability to adequately mimic the episodic secretion profile of testosterone by our replacement regimen or the contribution of another, as yet unidentified, gonadal androgen to PKC\textsubscript{δ} regulation. Additional studies will be needed to completely describe the regulation of PKC\textsubscript{δ} by male sex hormones. However, our in vitro findings with testosterone and DHT clearly support a prominent role of androgens in stimulating coronary smooth muscle PKC\textsubscript{δ} expression and activity. Together these studies demonstrate potential species- and vascular bed-specific effects of sex hormones that should be considered when interpreting sex comparisons. L-type, voltage-gated Ca\textsuperscript{2+} calcium channels are heteromeric complexes minimally composed of three protein subunits, α\textsubscript{1}, α\textsubscript{2}/δ, and β. The α\textsubscript{1} subunit (Ca\textsubscript{v}1.2) forms the channel pore and contains the binding sites for dihydropyridine antagonists. L-type Ca\textsuperscript{2+} channels mediate calcium entry into smooth muscle cells and play a central role not only in excitation-contraction coupling but also in gene expression and differentiation (23, 24). Our previous studies report a stimulatory effect of testosterone on Ca\textsubscript{v}1.2 mRNA and protein levels (11, 12), suggesting a transcriptional and translational stimulation of the Ca\textsubscript{v}1.2 gene by testosterone. Accordingly, Liu et al. (25) showed a hormone-responsive element in 5\textsuperscript{′} flanking region activated by testosterone in cardiac and smooth muscle cells. The present study involving various PKC isoform inhibitors and siRNA clearly shows a regulation of Ca\textsubscript{v}1.2 protein levels through the novel isoform PKC\textsubscript{δ}. Rottlerin, a putative PKC\textsubscript{δ} inhibitor, and chelerythrine, a generalized PKC inhibitor, blocked the testosterone-induced increase in Ca\textsubscript{v}1.2 protein levels. In contrast, Go6976, a conventional PKC isoform inhibitor, had no effect on testosterone stimulation of Ca\textsubscript{v}1.2 protein. Furthermore, failure of testosterone to increase Ca\textsubscript{v}1.2 protein levels in the presence of PKC\textsubscript{δ} siRNA con-
firmed the mandatory role of PKCδ in testosterone-induced Ca,1.2 protein up-regulation.

Although the present study is the first to report PKCδ-dependent up-regulation of Ca,1.2 protein in smooth muscle, these findings are consistent with PKCδ-dependent up-regulation of Ca,1.2 protein by ethanol in PC12 cells (26, 27). Whether PKCδ acts via a translational or post-translational mechanism is unknown. The Ca,1.2 promoter region contains numerous cis-acting response elements, including Nkx2.5, CRE-BP, HRE, CREBp and AP-1 (25), while PKCδ has been shown to stimulate AP-1/Jun-responsive genes (28). However, in PC12 cells, up-regulation of Ca,1.2 protein, but not mRNA, was PKCδ dependent (26), indicating a post-translational regulation of Ca,1.2 by PKCδ in these cells. We previously reported that testosterone increases both mRNA and protein levels of Ca,1.2 in coronary smooth muscle (12); however, it remains unknown whether the obligatory role of PKCδ in testosterone-induced increases in Ca,1.2 protein is post-translational or via increased Ca,1.2 gene expression.

Most peripheral tissues express aromatase and 5α-reductase, which convert testosterone to 17β-estradiol or dihydrotestosterone, respectively, allowing tissue-specific control over the immediate hormonal milieu. Local conversion of testosterone to estrogen via aromatase has been proposed to mediate testosterone effects in the brain (29), vascular smooth muscle cells, and ovary (30). Porcine coronary smooth muscle and endothelium express both androgen and estrogen receptors (12, 31). Our finding in this study that DHT produced up-regulation of PKCδ protein and activity levels similar to testosterone demonstrates that aromatization of testosterone to estrogen (31, 33) is not necessary for up-regulation of PKCδ protein levels, activity, and PKCδ-mediated Ca,1.2 protein levels of both testosterone and DHT.

The up-regulation of PKCδ by testosterone in the present study likely contributes to increased Ca,1.2 protein and activity levels in coronary arteries of males compared with females (11, 12), providing a novel and important mechanism for sex differences in coronary pathophysiology. For example, testosterone-mediated increases in coronary smooth muscle PKCδ may represent a primary mechanism underlying recent clinical evidence that physiological levels of testosterone protect against atherosclerosis in males (5–7). PKCδ-deficient mice show enhanced atherosclerotic lesion development in vein grafts, primarily because of enhanced smooth muscle cell infiltration (34), consistent with anti-proliferative, pro-apoptotic effects of PKCδ. Therapeutically, maintenance of normal endogenous testosterone in males may retard the progression of atherosclerotic lesion development by maintaining optimal levels of PKCδ in coronary smooth muscle.

In conclusion, the present study clearly demonstrates that testosterone is a primary modulator of PKCδ protein activity and PKCδ-dependent increases in Ca,1.2 protein expression in coronary smooth muscle. These studies provide the first mechanistic data linking testosterone regulation of Ca,1.2 protein levels to PKCδ. Recent studies have demonstrated that both Ca,1.2 and PKCδ control smooth muscle cell contraction, differentiation, and proliferation (23, 32). The present study provides the foundation for future studies to determine the effect of testosterone on coronary smooth muscle phenotype modulation and, ultimately, whether these factors contribute to the apparent salutary effect of testosterone on the development of coronary artery disease.

Acknowledgments—We thank Dr. Venkatesh Ganjani, Dr. Leona Rubin, Denise Holiman, and Dr. Vamsidhara Dhulipala for invaluable contributions to this study.

REFERENCES

1. American Heart Association (2003) Heart Disease and Stroke Statistics-Update, Dallas
2. Alexandersen, P., Haarbo, J., and Christiansen, C. (1996) Atherosclerosis 125, 1–13
3. Barrett-Connor, E., and Khaw, K. T. (1988) Circulation 78, 539–545
4. Helli, R. F., Jacobs, H. S., Vermeulen, A., and Deshpere, J. P. (1981) Br. Med. J. Clin. Res. Ed. 282, 438–439
5. Sieminska, L., Wojciechowska, C., Swietochowska, E., Marek, B., Kot-Kudla, B., Kajdaniuk, D., and Nowalanzy-Kozioleka, E. (2003) Med. Sci. Monit. 9, CR162-CR166
6. Dunaiajska, K., Milewicz, A., Szmuczyk, J., Jedrzejuk, D., Kuleczkowski, W., Salomon, P., and Nowicki, P. (2004) Aging Male 7, 197–204
7. Fukui, M., Kitagawa, Y., Nakamura, N., Kadono, M., Mogami, S., Hirata, C., Ichino, N., Wada, K., Hasegawa, G., and Yoshikawa, T. (2003) Diabetes Care 26, 1869–1873
8. Karas, R. H., Patterson, B. L., and Mendelsohn, M. E. (1994) Circulation 89, 1943–1950
9. Higashihara, K., Mathur, R. S., and Halushka, P. V. (1997) J. Cardiovasc. Pharmacol. 29, 311–315
10. Ma, R., Wu, S. Z., and Lin, Q. S. (2005) J. Appl. Physiol. 91, 2503–2510
11. Bowles, D. K. (2001) J. Appl. Physiol. 91, 2503–2510
12. Bowles, D. K., Maddali, K. K., Ganjam, V. K., Rubin, L. J., Tharp, D. L., Turk, J. R., and Heaps, C. L. (2004) Am. J. Physiol. 287, H2091-H2098
13. Andrea, J. E., and Walsh, M. F. (1992) Hypertension 20, 585–595
14. Khalil, R. A., Lajoie, C., Resnick, M. S., and Morgan, K. G. (1992) Am. J. Physiol. 263, C714-C719
15. Itoh, H., Yamamura, S., Ware, J. A., Zhuang, S., Mii, S., Liu, B., and Kent, K. C. (2001) Am. J. Physiol. 281, H359-H370
16. Obejeo-Paz, C. A., Auslander, M., and Scarpa, A. (1998) Am. J. Physiol. 275, C535-C543
17. Kanashiro, C. A., and Khalil, R. A. (2001) Am. J. Physiol. 280, C34-C45
18. Korzick, D. H., Rishel, M. E., and Bowles, D. K. (2005) Med. Sci. Sports Exerc. 37, 381–388
19. Hill, B. J., Katwa, L. C., Warnhoff, B. R., and Sturek, M. (2000) Am. J. Physiol. 295, 484–491
20. Maddali, K. K., Korzick, D. H., Turk, J. R., and Bowles, D. K. (2005) Vascul. Pharmacol. 42, 153–162
21. Hanke, H., Lenz, C., Hess, B., Spinellier, K. D., and Weidemann, W. (2001) Circulation 103, 1382–1385
22. Litten, R. Z., Suba, E. A., and Roth, B. L. (1987) Eur. J. Pharmacol. 144, 185–191
23. Warnhoff, B. R., Bowles, D. K., McDonald, O. G., Sinha, S., Somlyo, A. P., Somlyo, A. V., and Owens, G. K. (2004) Circ. Res. 95, 406–414
24. Nelson, M. T., Patlak, J. B., Worley, J. F., and Staben, N. B. (1990) Am. J. Physiol. 259, C3-C18
25. Liu, L., Fan, Q. I., El Zaru, M. R., Vanderpool, K., Hines, R. N., and Marsh, J. D. (2000) Am. J. Physiol. Heart 278, H1153-H1162
26. Walter, H. J., McMahon, T., Dedgar, J., Wang, D., and Messing, R. O. (2000) J. Biol. Chem. 275, 25717–25722
27. Gerstein, E. H., Jr., McMahon, T. J., and Messing, R. O. (1998) J. Biol. Chem. 273, 16409–16414
28. Hirai, S., Izumi, Y., Higa, K., Kabuchi, K., Mizuno, K., Osada, S., Suzuki, K., and Ohno, S. (1994) EMBO J. 13, 2331–2340
29. Balthazar, J., and Foidart, A. (1993) J. Steroid Biochem. Mol. Biol. 44, 521–540
30. Harada, N., Sasano, H., Murakami, H., Okuma, T., Nagura, H., and Takagi, Y. (1999) Circ. Res. 84, 1285–1291
31. Nathan, L., Shi, W., Dinh, H., Mukherjee, T. K., Wang, X., Luiz, A. J., and Chaudhuri, G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3589–3593
32. Mayr, M., Siow, R., Chung, Y. L., Mayr, U., Griffiths, I. J., and Xu, Q. (2004) Circ. Res. 94, 878–969
33. Mukherjee, T. K., Dinh, H., Chaudhuri, G., and Nathan, L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4055–4060
34. Leitges, M., Mayr, M., Braun, U., Mayr, U., Li, C., Pfister, G., Ghaffari-Tabarzi, N., Baier, G., Hu, Y., and Xu, Q. (2001) J. Clin. Investig. 108, 1505–1512