Comparison of the involvement of protein kinase C in agonist-induced contractions in mouse aorta and corpus cavernosum

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ABSTRACT

Protein kinase C (PKC) is involved in the regulation of vascular smooth muscle contraction. However, the role of PKC in erectile function is poorly understood. This study investigated whether PKC mediates agonist-induced contractions in mouse penile tissue (corpora cavernosa). We also compared the effects of PKC activators and inhibitors on contractile responses in mouse corpus cavernosum with those in mouse aorta. Aortic rings and corpus cavernosal strips from C57BL/6J mice were mounted in the organ bath for isometric tension recording. Our data showed that a $PKC_{\alpha/\beta}$ selective inhibitor, $G_{\ddot{o}}6976$ (10 μM), inhibited phenylephrine and 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619, a thromboxane mimetic)-induced contractions in mouse aorta, reducing the maximum contraction by 94% and 17%, respectively. A non-selective PKC inhibitor, chelerythrine (30 μM), also significantly reduced phenylephrine- and U46619-induced maximum contractions in mouse aorta. However, $G_{\ddot{o}}6976$ and chelerythrine had no significant effects on phenylephrine- and U46619-induced contractions in corpus cavernosum. Furthermore, a PKC activator, phorbol-12,13-dibutyrate (0.1 μM), significantly increased contractions in aorta (208 ± 14% of KCl-induced maximum contraction) but failed to cause contractions in corpus cavernosum at 1 and 10 μM. Western blot analysis data suggested that protein expression of PKC was similar in aorta and corpus cavernosum. Taken together, our data indicate that PKC does not have a significant role in agonist-induced contractions in mouse corpus cavernosum, whereas it mediates the contractile response to agonists in the aorta.

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1. Introduction

Erectile dysfunction is recognized as a public health problem in recent years and increasing efforts have been sought to treat this disease. Vascular dysfunction is one of the common causes of erectile dysfunction. Corpus cavernosum has a specialized vasculature (Andersson and Wagner, 1995). It is composed of numerous interconnected cavernosal spaces, which are lined by a layer of endothelial cells and separated by trabeculae, containing smooth muscle fibers, elastic fibers and fibroblasts. Penile smooth muscle stays contracted in the flaccid state, which is dominated by sympathetic neuronal activity. Erection occurs when the penile arteries and corpus cavernosal smooth muscle are relaxed by nitric oxide released from nerve endings and endothelial cells. This relaxation allows blood to flow into the penis under the driving force of systemic blood pressure. Vasculogenic erectile dysfunction is often due to an imbalance of contractile and relaxation mechanisms. Under pathophysiological conditions, increased release of adrenergic neurotransmitters such as norepinephrine or circulating constrictive factors lead to an increase in smooth muscle tone of penile tissue (Disanto, 2005; Jin and Burnett, 2006).

Protein kinase C (PKC) is an important molecule that transduces extracellular stimuli to intracellular signal cascades. It is involved in the signaling pathways that regulate cell growth, apoptosis, ion channel activities, actin cytoskeleton, and carcinogenesis. Functional studies have demonstrated that PKC mediates agonist-induced contractions in various arteries (Budzyn et al., 2006). Moreover, evidence suggests that increased PKC activity causes hyperactive contractility of vascular smooth muscle, contributing to the development of cardiovascular diseases (Salamanca and Khalil, 2005). Increased PKC activity in diabetic human penile tissue has also been reported (Angulo et al., 2006). PKC inhibitors may be a potential therapeutic treatment for vascular diseases.

Studies have demonstrated that rodents are valid animal models for the investigation of erectile physiology and pharmacology (Burnett, 2001; Giuliano, 2001). Although rats have been widely used for in vivo and in vitro assessments of erectile function, the mouse model has recently become attractive due to the accessibility of genetic manipulations. The knockout mouse models, such as nitric

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oxide synthase knockouts and cyclic GMP-dependent protein kinase knockouts, have provided valuable data at the molecular level on the specific signaling pathways involved in erection. However, there is a lack of information on physiological and pharmacological characterizations of mouse penile tissue. The functions of PKC in the regulation of smooth muscle tone in mouse penis are unknown. The goal of this study is to determine the involvement of PKC in mediating agonist-induced contractions in mouse corpus cavernosum and to compare its roles with those in mouse aorta, thereby providing basic information for future studies of erectile function in mice. We used the C57BL/6J mouse as our animal model since it is commonly used as the background strain for the genetic mutants.

2. Materials and methods

2.1. Animals and tissue preparation

C57BL/6J mice (8 to 10 weeks old) were purchased from Jackson Laboratory. The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes for Health.

The mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Aortae were rapidly excised and placed in cold physiological saline solution (PSS) of the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.18, $CaCl_2 \cdot 2H_2O$ 1.6, $MgSO_4 \cdot 7H_2O$ 1.6, $NaHCO_3$ 25, dextrose 5.5, EDTA 0.03. After periadventitial adhering fat was removed, endothelial intact aortae were cut into 2 mm rings and mounted in myograph organ bath chambers (Danish Myo Technology A/S).

Mice penes were exposed and excised from skin and connective tissue. After removal of spongiosum and dorsal vein and arteries in cold PSS, mouse corpora cavernosa were cut longitudinally to obtain two identical strips, which were mounted on organ bath chambers.

2.2. Experimental protocols

The aortic rings or cavernosal smooth muscle strips were placed under an optimal passive tension of 5 mN or 2 mN, respectively, and allowed to equilibrate for 60 min in 5 ml PSS continuously bubbled with 95% O_2 and 5% CO_2 at 37 °C. After equilibration, aortic rings or cavernosal muscle strips were contracted to the maximum with 120 mM KCl to verify the viability of the preparations. After washing out KCl, the aortic rings or cavernosal strips were incubated with vehicle (0.1% DMSO), $G_ö6976$ (a selective $PKC_{\alpha/\beta}$ inhibitor), or chelerythrine (a non-selective PKC inhibitor) in the absence or presence of N(G)-nitro-L-arginine methyl ester (L-NAME) for 20 min. Cumulative concentration–response curves to phenylephrine (an α-adrenoceptor agonist) or 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$ (U46619, a thromboxane mimetic) were constructed. In another set of experiments, phorbol-12,13-dibutyrate (PDBu) was added into organ bath chambers after verification of the viability of the aortic rings and cavernosal strips. The contractile responses to PDBu were monitored for 45 min after the administration of the chemical.

Fig. 1. Effects of PKC inhibitors on agonist-induced contraction in mouse aorta. (A) Pre-treatment of $G_ö6976$ decreased phenylephrine-induced contraction (■: control; ●: pre-treated with 1 μM of $G_ö6976$; ▲: pre-treated with 10 μM of $G_ö6976$). $n=4$, ** $P<0.01$ vs. control. (B) Pre-treatment of chelerythrine reduced the contractile responses to phenylephrine (■: control; ●: pre-treated with 10 μM of chelerythrine; ▲: pre-treated with 30 μM of chelerythrine). $n=4$, ** $P<0.01$ vs. control. (C) $G_ö6976$ reduced the contractile response to U46619 at 10 μM but had no effects at 1 μM (■: control; ●: pre-treated with 1 μM of $G_ö6976$; ▲: pre-treated with 10 μM of $G_ö6976$). $n=4$, * $P<0.05$ vs. control. (D) Chelerythrine concentration-dependently decreased the contractions induced by U46619 (■: control; ●: pre-treated with 10 μM of chelerythrine; ▲: pre-treated with 30 μM of chelerythrine). $n=4$, ** $P<0.01$ vs. control.
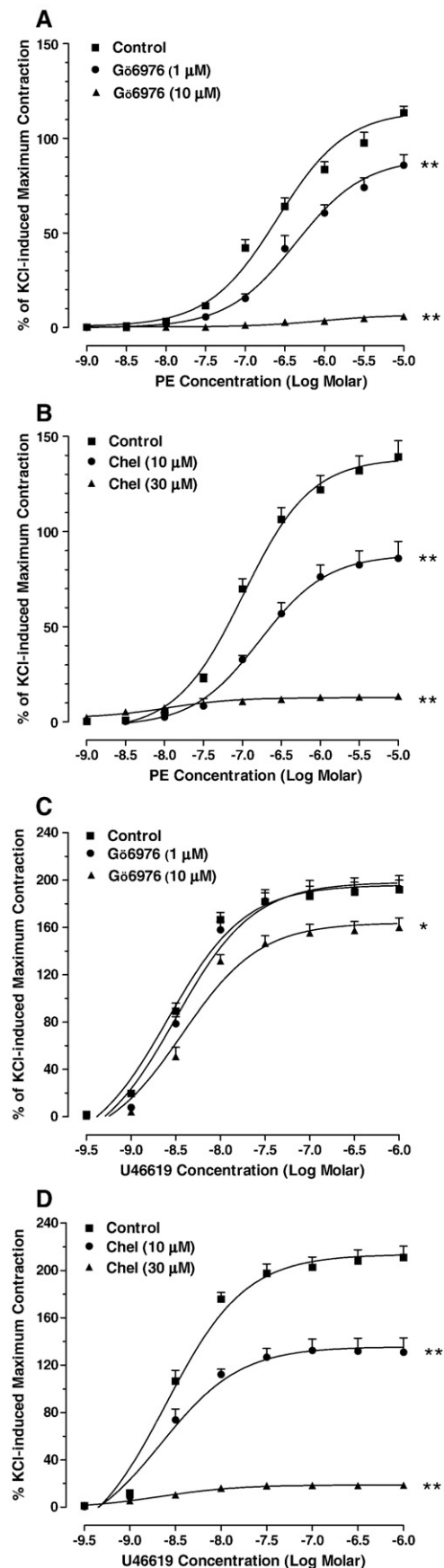
Changes in isometric force were recorded by PowerLab data acquisition system (ADInstruments) and expressed as % of KCl-induced maximum contraction.

2.3. Western blot analysis for PKC protein expression

Mouse aorta or corpus cavernosum was homogenized in RIPA buffer (Upstate) containing Tris–HCl, pH 7.4, 50 mM, NaCl 150 mM, deoxycholic acid 0.25%, NP-40 1%, EDTA 1 mM, phenylmethylsulfonyl fluoride 1 mM, and Na_3VO_4 1 mM. The tissue lysates were centrifuged at 10,000 g for 30 min at 4 °C and the supernatant was collected. Proteins (25 μg) were loaded on 7.5% SDS-polyacrylamide gel for Western blot analysis as described previously (Jin et al., 2006). Primary antibodies against PKC_α (1:1000 dilution) and PKC_β (1:500 dilution) isoforms (BD Biosciences) or β-actin (1:5000, Sigma) were used to detect PKC and β-actin protein expression in mouse aorta or corpus cavernosum. Protein expression of PKC_α or PKC_β was normalized by β-actin protein level.

2.4. Statistics

Data were expressed as the mean±S.E.M. EC_{50} was obtained from the sigmoidal dose–response curve fit. Analysis of variance and Student's t-test were used to evaluate the results. $P < 0.05$ was considered to be significant. GraphPAD Software was used for the statistical analysis of all data.

3. Results

3.1. Effects of PKC inhibitors on agonist-induced contraction in mouse aorta

Phenylephrine produced a concentration-dependent contraction curve in mouse aorta with EC_{50} equal to 0.25 μM. The PKC inhibitor $G_ö6976$ (1 μM) shifted the curve to the right and increased the EC_{50} of phenylephrine 1.7-fold (Fig. 1A). Additionally, the maximum contraction in response to phenylephrine was significantly reduced from 123±2% of KCl-induced maximum contraction to 92±5%. A higher concentration of $G_ö6976$ (10 μM) almost blocked the contractile response to phenylephrine, decreasing the maximum contraction to 7±2%. The inhibitory effect of another PKC inhibitor, chelerythrine, also occurred in a concentration-dependent fashion. Chelerythrine (10 μM) decreased phenylephrine-induced maximum contraction by 40% when compared to that of vehicle controls (Fig. 1B). At higher concentration (30 μM), chelerythrine almost abolished the phenylephrine-induced contraction.

We also determined the effects of PKC inhibitors on the thromboxane receptor agonist-induced contractions. U46619 induced concentration-dependent contractions in mouse aorta with EC_{50} equal to 0.003 μM. At 10 μM, $G_ö6976$ reduced the maximum smooth muscle contraction by 17% in response to U46619 and increased EC_{50} of U46619 to 0.004 μM in mouse aorta; however, it did not have a significant effect at 1 μM (Fig. 1C). Chelerythrine had profound effects on U46619-induced contractions, decreasing the maximum responses by 38% and 91% when compared to those effects in vehicle controls at 10 and 30 μM, respectively (Fig. 1D). The EC_{50} of U46619 was not significantly altered by chelerythrine. These data suggest that PKC mediates agonist-induced aortic smooth muscle contraction.
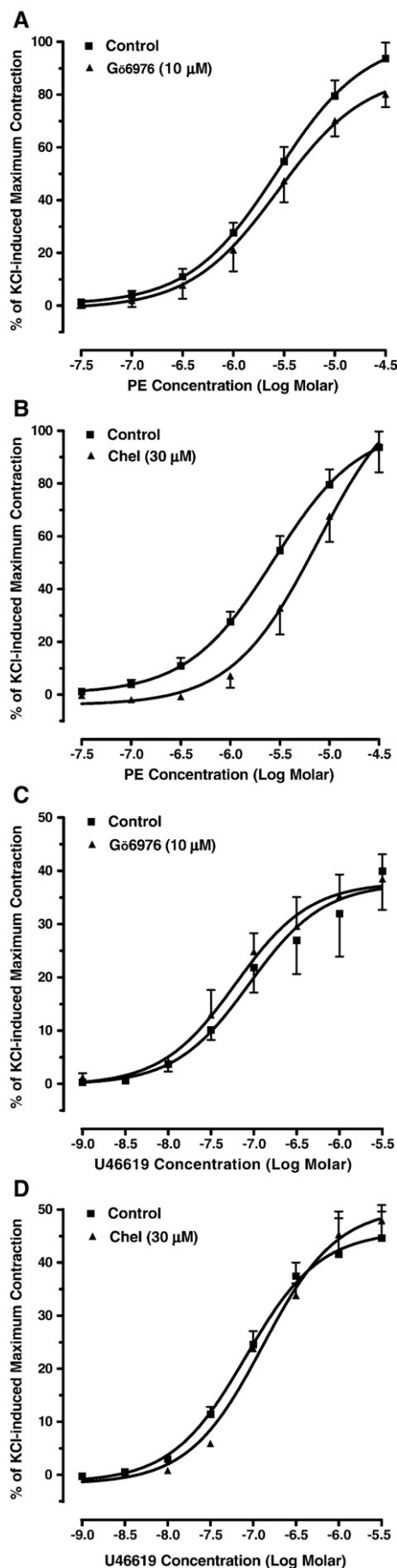

Fig. 2. Effects of PKC inhibitors on agonist-induced contraction in mouse corpus cavernosum. (A) Pre-treatment of $G_ö6976$ did not alter phenylephrine-induced contraction (■: control; ▲: pre-treated with 10 μM of $G_ö6976$). $n = 6$. (B) Pre-treatment of chelerythrine had no significant effect on the contractile responses to phenylephrine. (■: control; ▲: pre-treated with 30 μM of chelerythrine). $n = 4$. (C) $G_ö6976$ had no effect on U4419-induced contraction (■: control; ▲: pre-treated with 10 μM of $G_ö6976$). $n = 4$. (D) Chelerythrine had no effect on the contractions induced by U44619 (■: control; ▲: pre-treated with 30 μM of chelerythrine). $n = 4$.
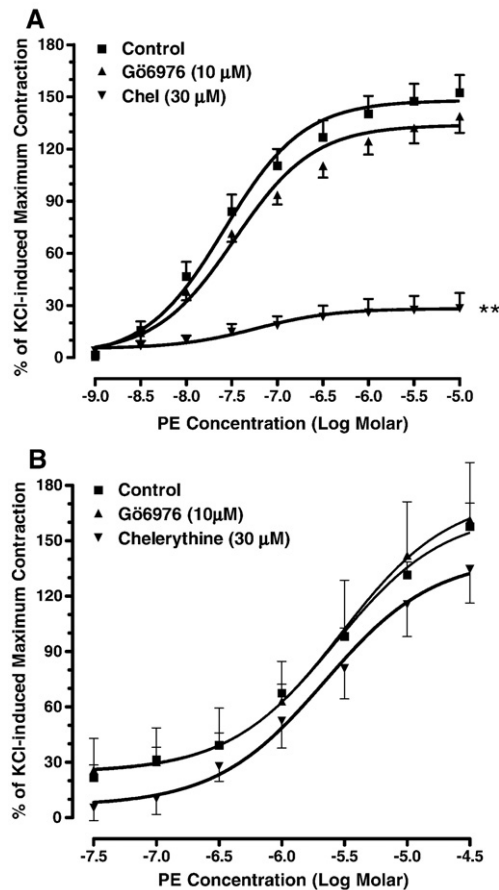
Fig. 3. Effects of PKC inhibitors on phenylephrine-induced contraction in the presence of L-NAME. (A) Aortic rings were pre-incubated with L-NAME (500 μM) only (■), L-NAME with $G_ö6976$ (▲), or L-NAME with chelerythrine (▼) for 20 min. $n=4$, ** $P<0.01$ vs. control. (B) Corpus cavernosal smooth muscle strips were pre-incubated with L-NAME (500 μM) only (■), L-NAME with $G_ö6976$ (▲), or L-NAME with chelerythrine (▼) for 20 min. $n=4$.

3.2. Effects of PKC inhibitors on agonist-induced contraction in mouse corpus cavernosum

To investigate the role of PKC in mouse erectile function, we examined the effects of PKC inhibitors on phenylephrine or U46619-induced contractions in mouse corpus cavernosal smooth muscle. Phenylephrine elicited a concentration-dependent contraction curve in mouse cavernosal muscle strip with an EC_{50} of 0.27 μM. The maximum response to phenylephrine is $94±6\%$ of KCl-induced contraction. $G_ö6976$ failed to alter the contractile actions of phenylephrine at the concentration (10 μM) that abolished the phenylephrine-induced contraction in mouse aorta (Fig. 2A). Pre-incubation with chelerythrine (30 μM) also had no significant effect on the contractions caused by phenylephrine (Fig. 2B). Moreover, the contractile responses to U46619 ($EC_{50}=0.081$ μM) were unchanged in the corpus cavernosal smooth muscle strips in the presence of $G_ö6976$ (10 μM) and chelerythrine (30 μM) (Fig. 2C and D).

3.3. Effects of PKC inhibitors on mouse aorta and corpus cavernosum in the presence of L-NAME

Nitric oxide (NO), a major vasodilator, is constantly produced by endothelial cells in the aorta and the penis. To investigate whether the basal level of NO production influences the efficacy of PKC inhibitors, mouse aortic rings or cavernosal smooth muscle strips were pre-incubated with either L-NAME (500 μM) only or L-NAME plus a PKC

inhibitor. In mouse aorta, pre-incubation of L-NAME significantly increased phenylephrine-induced maximum contraction ($126±8\%$ of control, $P<0.01$, data not shown). The inhibitory effects of $G_ö6976$ (10 μM) and chelerythrine (30 μM) on phenylephrine-induced contraction were markedly decreased in the presence of L-NAME (Fig. 3A). However, L-NAME did not affect phenylephrine-induced maximum contraction in mouse cavernosal smooth muscle strips ($99±5\%$ of control, data not shown). This suggests that unlike in aorta, the basal release of NO in cavernosal strips is insignificant. $G_ö6976$ or chelerythrine did not alter the contractile response to phenylephrine in cavernosal smooth muscle in the presence of L-NAME (Fig. 3B), suggesting that NO does not cause the lack of inhibitory effects by the PKC inhibitors.

3.4. Effects of a PKC activator on mouse aorta and corpus cavernosum

To further compare the role of PKC in mediating contractions in mouse aorta and corpus cavernosum, the effects of a specific activator of PKC, PDBu, were examined. The addition of PDBu (0.1 μM) caused a strong and sustained contraction in the aorta, and the maximum contraction reached to $208±14\%$ of KCl-induced maximum contraction. However, PDBu failed to induce contraction in the cavernosal smooth muscle strips even when the concentrations were increased to 1 or 10 μM (Fig. 4).

3.5. PKC protein expression in mouse aorta and corpus cavernosum

To determine whether the PKC's differential mediation of agonist-induced contractions in mouse aorta and corpus cavernosum is due to different levels of protein content, we determined and compared PKC protein expression in those tissues. Data from Western blot analysis suggested that both $PKC_α$ and $PKC_β$ were expressed in the aorta and
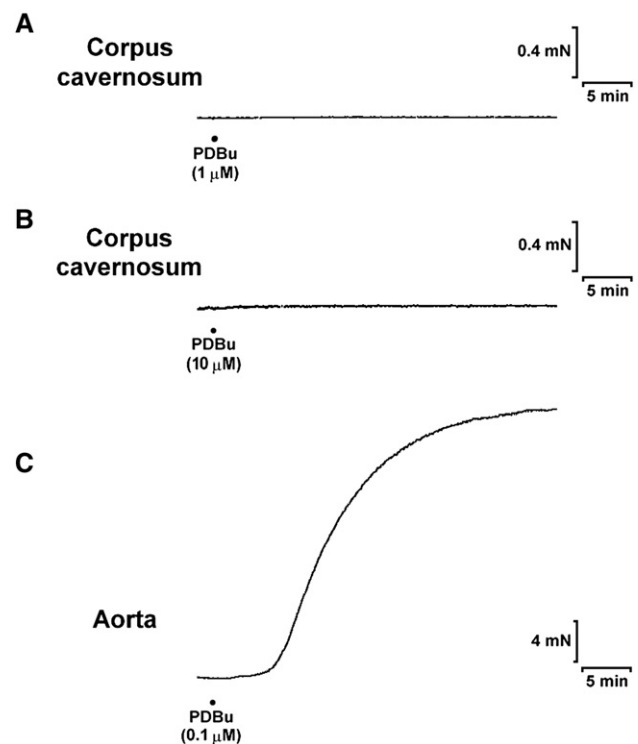

Fig. 4. Effects of PDBu on the contractility of mouse aorta and corpus cavernosum. Panels (A) and (B) show the representative tracings obtained in corpus cavernosal strips after stimulation with PDBu at 1 and 10 μM, respectively. PDBu failed to cause contractions at both concentrations. $n=4$. Panel (C) shows the representative tracing obtained in aortic rings after stimulation with 0.1 μM of PDBu. The maximum contraction is $208±14\%$ KCl-induced contractions. $n=4$.
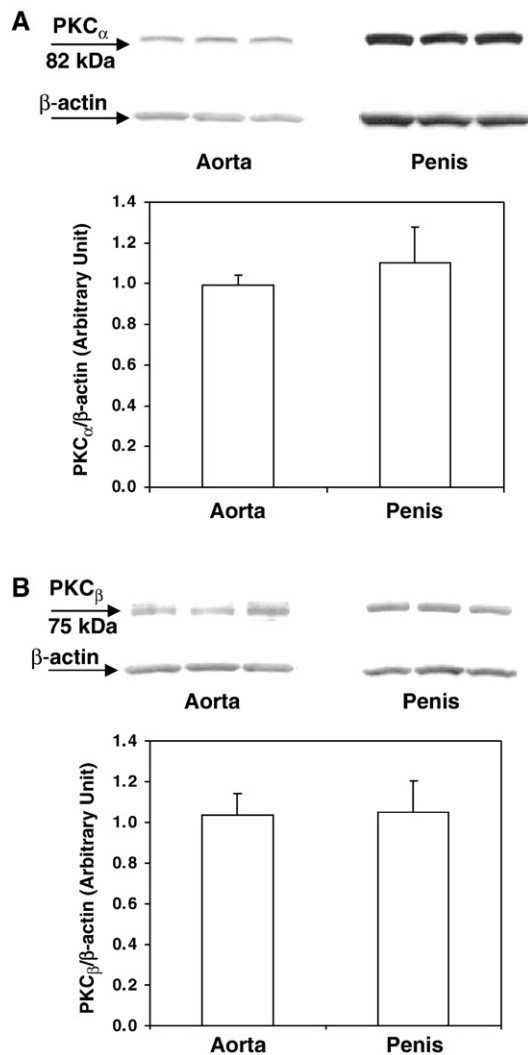
Fig. 5. PKC protein expressions in mouse aorta and corpus cavernosum. (A) A representative immunoblot of PKC_α protein expression from 3 mice and the summary of data. $n=5$. (B) A representative immunoblot of PKC_β protein expression from 3 mice and the summary of data. $n=7$. Protein expressions of PKC were normalized by β-actin level.

corpus cavernosum. The levels of PKC protein expression were similar in mouse aorta and corpus cavernosum after normalization by β-actin protein level (Fig. 5).

4. Discussion

In response to receptor activation, contraction in most types of smooth muscle involves an increase in intracellular Ca^{2+} levels and enhancement of Ca^{2+} sensitivity. Activation of G protein coupled receptor by agonists increases phospholipase activity and sequentially inositol phosphoholipid hydrolysis, leading to the formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases intracellular Ca^{2+} release from sarcoplasmic reticulum, which thereby increases Ca^{2+}-dependent PKC activity; meanwhile, DAG is able to activate PKC through direct physical binding. Evidence suggests that PKC mediates Ca^{2+} sensitization pathways and increases smooth muscle contraction either by inhibition of myosin light chain phosphatase or by inhibition of actomyosin ATPase (Somlyo and Somlyo, 2003; Wier and Morgan, 2003).

The study by Budzyn et al. (2006) suggests that the degree to which PKC is involved in contractile response depends upon the sizes of arteries. The contribution of PKC to agonist-induced contractions is increased when the size of arteries is decreased. In our study, we compared the roles of PKC in mediating contractile response in different tissues. We found that PKC inhibitors significantly decreased phenylephrine- and U46619-induced contractions in mouse aorta, suggesting an important role for PKC in mediating aortic smooth muscle contraction. However, $PKC_{\alpha/\beta}$ may play a less significant role in U46619-induced contraction, since $G_\delta 6976$, a $PKC_{\alpha/\beta}$ selective inhibitor, was less effective in reducing the contractions. Consistent with our observations, previous studies have shown that PKC mediates the contractile responses upon agonist stimulations in various arteries, including aorta, mesenteric, femoral, and tail arteries isolated from rat, canine, or guinea pig (Jiang et al., 1994; Merkel et al., 1991; Sato et al., 2001; Shima and Blaustein, 1992). In addition, data from us and other groups show that PKC activators have potent contractile effects on aortic smooth muscle. It has been suggested that abnormally enhanced PKC activity contributes to increased vascular reactivity in disease states. For example, the response to phorbol esters is greater in the arteries from hypertensive animals as well as diabetic animals when compared to those in their normal counterparts (Bilder et al., 1990; Suzuki et al., 1994; Turla and Webb, 1987, 1991; White and Carrier, 1990). In diabetic mouse aorta, increased phenylephrine-induced contractions were abolished by chronic treatment with a PKC_β selective inhibitor, suggesting that PKC activity is responsible for the increased agonist-induced contraction in diabetic aorta (Nangle et al., 2003).

On the other hand, little is known about the role of PKC in penile tissue, such as the effects of PKC on agonist-induced corpus cavernosal smooth muscle contraction. Studies done by Holmquist et al. (1990) showed that application of a PKC inhibitor, H7, abolished the endothelin-1-induced contraction in rabbit corpus cavernosum in the Ca^{2+} free medium. These observations indicate that PKC may be involved in Ca^{2+} sensitization mechanisms of endothelin-1-induced contraction. However, the authors concluded that the non-specificity of H7, which also inhibits cyclic AMP or cyclic GMP-dependent protein kinases, limited the value of these observations. Nangle et al. (2003) reported that the contractile responses to phenylephrine were unchanged in cavernosal muscle strips isolated from diabetic mice or diabetic mice chronically treated with a PKC_β inhibitor when compared to those from the control mice. Because there was no comparison made to the cavernosal muscle strips from control mice treated with the PKC_β inhibitor, it is not clear whether the lack of changes in phenylephrine-induced contractions in diabetic cavernosum or PKC_β inhibitor-treated diabetic cavernosum is because PKC does not mediate agonist-induced contractions in erectile tissue.

In the present study, we found that PKC inhibitors had no effects on phenylephrine- or U46619-evoked contractions and PDBu failed to induce contractions in mouse corpus cavernosal smooth muscle strip. Furthermore, inhibition of NO synthase by L-NAME did not change the contractile responses in the presence of PKC inhibitors. Cavernosal smooth muscle strips pre-incubated with L-NAME did not response to PDBu either (data not shown). These data suggest that PKC plays a minimal role in contractile responses to these agonists in mouse corpus cavernosum. Consistent with our findings, recent studies done by Angulo et al. (2006) showed that contractile response to U46619 in non-diabetic human cavernosal muscle was not altered by PKC inhibition. Interestingly, their results suggest that PKC is responsible for the increased contraction induced by U46619 in diabetic human cavernosal muscle because the PKC inhibitor completely reversed this hypersensitive response. Therefore, it is likely that PKC is not involved in the regulation of agonist-induced contraction in normal mouse or human cavernosal tissue, while activation of PKC may be an underlying mechanism for increased contractility of caverosal tissue in disease state.

In rat corpus cavernosum, PKC was suggested to mediate phenylephrine-induced contraction, yet the potency of the PKC inhibitor,

cheleythrine, was not compared between rat aorta and corpus cavernosum (Husain et al., 2004). The different effect of PKC on corpus cavernosal contraction probably is due to species difference.

In conclusion, our study provides the pharmacological characterizations of PKC in agonist-induced contractions in both mouse corpus cavernosum and mouse aorta. Our data suggest that activation of PKC is required for agonist-induced contractions in mouse aorta; however, PKC is not involved in corpus cavernosal smooth muscle contractile responses to the agonists. Further investigations are needed to examine the functions of PKC in penile tissue and its role in erectile dysfunction.

Acknowledgments

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