Journal of Cellular Biochemistry 80:367–372 (2001)

Translocation of PKC Isoforms in Bovine Aortic Smooth Muscle Cells Exposed to Strain

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Abstract Our laboratory has previously reported that the exposure of smooth muscle cells (SMC) to the cyclic strain results in significant stimulation of protein kinase C (PKC) activity by translocating the enzyme from the cytosol to the particulate fraction. We now sought to examine the strain-induced translocation of individual PKC isoforms in SMC. Confluent bovine aortic SMC grown on collagen type I-coated plates were exposed to cyclic strain for up to 100 s at average 10% strain with 60 cycles/min. Immunoblotting analysis demonstrates that SMC express PKC-α, -β and -ζ in both cytosolic and particulate fractions. Especially, PKC-α and -ζ were predominantly expressed in the cytosolic fraction. However, cyclic strain significantly ($P < 0.05$) increased PKC-α and -ζ in the particulate fraction and decreased in the cytosolic fraction. Thus, the cyclic strain-mediated stimulation of PKC activity in SMC may be due to the translocation of PKC-α and -ζ from the cytosolic to the particulate fraction. These results demonstrate that mechanical deformation causes rapid translocation of PKC isoforms, which may initiate a cascade of proliferation responses of SMC since NF-κB, which is involved in the cellular proliferation has been known to be activated by these PKC isoforms. J. Cell. Biochem. 80:367–372, 2001. © 2001 Wiley-Liss, Inc.

Key words: hemodynamic forces; vascular cells; signaling

Mechanical stimuli are important modulators of cellular function in tissues, particularly in the vascular cells. Vascular smooth muscle cells (SMC) in vivo are continuously subjected to complex biomechanical forces induced by the pulsatile flow of blood through the circulatory system. A key physical force experienced by vascular smooth muscle cells is repetitive deformation in the form of cyclic strain created by the pulse wave. Such repetitive strains are physiologically relevant to the modulation of cell morphology, function and proliferation in vascular SMC.

Numerous studies using in vitro systems have demonstrated that cyclic strain exerted important effects on phenotype and growth of a number of different cell types including SMC. Our laboratory previously demonstrated that cyclic strain stimulated collagen synthesis [Sumpio and Banes, 1988] and elastin production [Costa et al., 1991] in porcine aortic SMC. In addition, SMC subjected to cyclic strain reorganized cytoskeleton [Brozovich, 1995; Mills et al., 1997] and altered proliferation [Sumpio and Banes, 1998; Brozovich, 1995; Mills et al., 1997; Wilson et al., 1993] and synthesis of macromolecules, such as interleukin 6 [Sumpio and Kupper, 1989], and Na^+/K^+-ATPase [Songu et al., 1996]. However, the pathways that couple the external physical forces and the cellular response are not well defined. Our laboratory has recently reported that cyclic strain rapidly activates PKC activity in aortic SMC by translocating the enzymes from the cytosol to the particulate fraction [Mills et al., 1997]. Thus, in this study, we examined whether cyclic strain activates individual PKC isoforms in SMC. We especially studied PKC-α and -β isoforms because the activation of PKC-α and -β has been known in other cell system, such as endothelial cells, in response to cyclic strain [Rosales and Sumpio, 1992]. In addition, we also determined whether cyclic strain also stimulates PKC-ζ isoform in SMC. PKC-ζ isoform, phorbol ester/DAG insensitive atypical PKC isoform, has been known to be critically important for the regulation of

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Received 11 July 2000; Accepted 12 July 2000

This article published online in Wiley InterScience, November XX, 2000.

function and proliferation in SMC [Nakanishi et al., 1993; Hoshi et al., 2000].

METHODS

Cell Culture

Bovine aortic SMC were obtained as explants of bovine thoracic aorta [Sumpio and Banes, 1988] and utilized at less than five passages. SMC were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin, and 2.8 mM L-glutamine. SMC were confirmed by their typical morphology and hill-and-valley pattern and by positive immunofluorescence with a monoclonal antibody directed against smooth muscle derived α-actin (Sigma, St. Louis, MO).

Application of Cyclic Strain In Vitro

SMC were grown to confluence on flexible-bottomed culture plates coated with type I collagen (Flex I plate, Flexcell, McKeesport, PA). The plates were positioned on a vacuum manifold, which was situated in a tissue culture incubator (5% CO_2, 37°C). The applied vacuum was regulated by solenoid valve controlled by a computer program. The strain apparatus (Flexercel, Flexcell) has been previously described and characterized in detail [Sumpio et al., 1987]. When vacuum is applied to the culture plates, the bottoms are deformed to a known percent elongation. When vacuum is released, the plate bottoms return to their original conformation. For these experiments, the flexible membranes were deformed with a 150-mmHg vacuum (10% average strain) at 60 cycles/min (i.e., 0.5 s elongation alternating with 0.5 s relaxation) for up to 100 s.

Detection of PKC Isoforms by Western Blotting

SMC were subjected to strain for up to 100 s, and then cells were harvested after the initiation of cyclic strain at various time points as described. Unstretched cells (time = 0) were used as controls. Cells were fractionated into the cytosolic and particulate fractions as previously described [Han et al., 1998]. Briefly, SMC were fractionated into the cytosolic and particulate fractions by ultracentrifugation in buffer containing 25 mM/l Tris–HCl, pH 7.6, 5 mM/l EGTA, 0.7 mM/l $CaCl_2$, 1 mM/l PMSF, 10 μM/l leupeptin. The particulate fraction includes both membrane and Triton X-100 soluble cytoskeletol. Protein concentration was determined by the Bradford method [Bradford, 1976] using the Bio Rad protein assay system (Bio Rad, Hercules, CA) taking BSA (Pierce, Rockford, IL) as a standard. Samples (20 μg protein) were boiled in Laemmli SDS buffer (60 mM Tris–HCl, 20% glycerol, 2% SDS, 10 mM β-mercaptoethanol, 0.2% bromophenol blue) for 5 min. SDS-treated samples were separated by 7.5% SDS-PAGE gel and electroblotted to nitrocellulose membranes [Towbin et al., 1979]. To block nonspecific antibody binding, the blots were incubated in TBST (50 mM Tris–HCl, 150 mM NaCl and 0.05% Tween-20, pH 7.4) containing 5% nonfat dry milk at room temperature for 1 h. The blots were then incubated with primary antibodies, either PKC-α (Upstate Biotechnology, Lake Placid, NY), PKC-β (Seikagaku Kogyo Co., Ltd., Tokyo, Japan), or PKC-ζ (Gibco BRL, Gaithersburg, MD) antibody in TBST for 1 h at room temperature. The PKC-α, -β, and -ζ were detected by using ECL chemiluminescent assay (Amersham, Arlington Heights, IL) following incubation with an appropriate peroxidase-coupled secondary antibodies and then quantitated by densitometric analysis using ImageQuant soft computer program (Molecular Dynamic, Sunnyvale, CA).

Data Analysis

Data are means ± SE. Data were analyzed using a paired *t*-test with a Bonferroni correction. A P value > 0.05 was considered as a statistically significant difference.

RESULTS

Characterization of PKC Isoforms in Bovine Aortic SMC

All three PKC isoforms, α, β, and ζ were detected in the cytosolic and particulate fractions of SMC (Fig. 1A) using monospecific antibodies against PKC-α, -β, and -ζ isoforms. The ratio of content of each isoform in the cytosolic and particulate fraction is shown in Figure 1B. The ratio of PKC-β was comparable between the cytosolic (60%) and particulate (40%) when the same amount of protein was applied. In contrast, PKC-α (85%) and -ζ (75%) were distributed preferentially in the cytosol fraction.

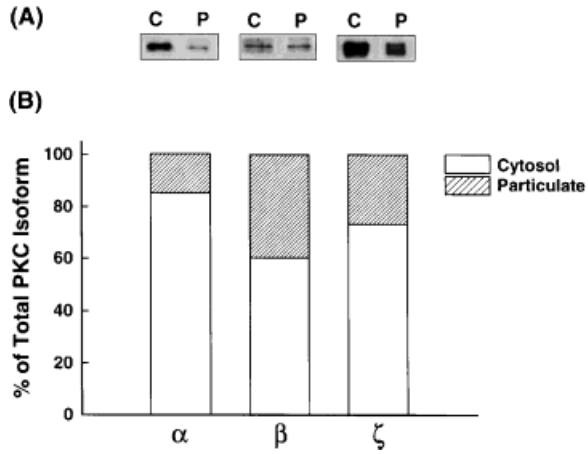

Fig. 1. Subcellular distribution of PKC isoforms in bovine aortic smooth muscle cells. Confluent SMC grown on collagen type I-coated plates were harvested and fractionated into cytosol and particulate fractions as described in Methods. The expression of individual PKC isoforms was assessed by immunoblotting with isotype specific antibodies. Same amount of protein was applied for each isoform in the cytosol and particulate fractions. **A**: Representative blots for PKC-α, -β and -ζ isoforms in cytosol (C) and particulate (P) fractions are from three independent experiments. **B**: Densitometric analysis of blots for PKC isoforms in the cytosol and particulate fraction. Values are expressed as % of total.

Translocation of PKC Isoforms in SMC Subjected to Cyclic Strain

The effect of cyclic strain on the redistribution of PKC isoforms in SMC was characterized. Since our laboratory previously showed that cyclic strain increased the membrane PKC activity by threefold within 1 min in SMC [Mills et al., 1997], we examined the distribution of PKC isoforms from 10 to 100 s after initiation of strain. Western blot analysis showed that cyclic strain increased PKC-α and -ζ isoforms in the particulate fraction up to 100 s (Fig. 2A). The peak of PK-α isoform in the particulate fraction occurred at 100 s after initiation of strain, and the peak of PKC-ζ isoform in the particulate fraction appeared at 20 s after exposure to strain (Fig. 2B). While PKC-α gradually increased in the particulate fraction from 10 to 100 s after initiation of strain, PKC-ζ rapidly increased in the particulate fraction. Densitometric analysis demonstrated that PKC-α increased ($P < 0.05$, $n = 3$) by $72 \pm 22\%$ in the particulate fraction and decreased ($P < 0.05$, $n = 3$) by $28 \pm 8\%$ in the cytosolic fraction in SMC exposed to cyclic strain for 100 s (Fig. 2B). Similarly, densitometric analysis of the blots showed that PKC-ζ increased ($P < 0.05$, $n = 3$) by 89 ± 27 and $69 \pm 15\%$ in the particulate fraction and decreased by 3 ± 4 ($P > 0.05$, $n = 3$) and $22 \pm 3\%$ ($P < 0.05$, $n = 3$) in the cytosol fraction, respectively, in SMC subjected to cyclic strain for 20 and 100 s, respectively (Fig. 2B).

While cyclic strain for 100 s increased the level of PKC-ζ isoform in the particulate fraction with a corresponding decrease in the cytosolic fraction, the increase of this isoform in the particulate fraction after 20 s exposure to cyclic strain occurred without a corresponding decrease in the cytosolic fraction. In contrast to the significant changes of PKC-α and -ζ isoforms in the particulate fraction, there was a minimal change in the distribution of PKC-β in the particulate ($5 \pm 29\%$ increase, $P > 0.1$, $n = 3$) and cytosolic fraction ($20 \pm 10\%$ decrease, $P > 0.1$, $n = 3$) in SMC subjected to cyclic strain for 100 s (Fig. 2B). In addition, cyclic strain for 10 s demonstrated decreased levels of PKC-α, -β and -ζ isoforms in the cytosolic fraction by 13–19% although these changes were not significant ($P > 0.1$, $n = 3$). The decreased levels of PKC-α and -β in the cytosolic fraction were much higher than the increased levels of these isoforms in the particulate fraction. Similarly, the decreased levels of both PKC-α and -β in the cytosolic fraction after 100 s exposure to strain were much higher than the increased levels of these isoforms in the particulate fraction indicating the possibility that cyclic strain translocates these PKC isoforms to the particulate fraction, or that cyclic strain may stimulate degradation of these isoforms.

DISCUSSION

The present study provides first observation that mechanical strain stimulates translocation of both Ca^{2+}-dependent and Ca^{2+}-independent PKC isoforms from the cytosolic to the particulate fraction in bovine aortic SMC. Immunoblot analysis demonstrates that PKC-α and -ζ isoforms are the isoforms responsible for mechanical force-induced PKC translocation from the cytosol to the particulate fraction in SMC.

Previous studies suggested that SMC express at least 5 PKC isoforms, -α, -β (βI and βII), -δ, -ε, -ζ [Assender et al., 1994; Khalil et al., 1992; Paul et al., 1997; Sasaguri et al., 1993] with predominant expression of PKC-α and -ζ isoforms [Assender et al., 1994; Paul et al.,

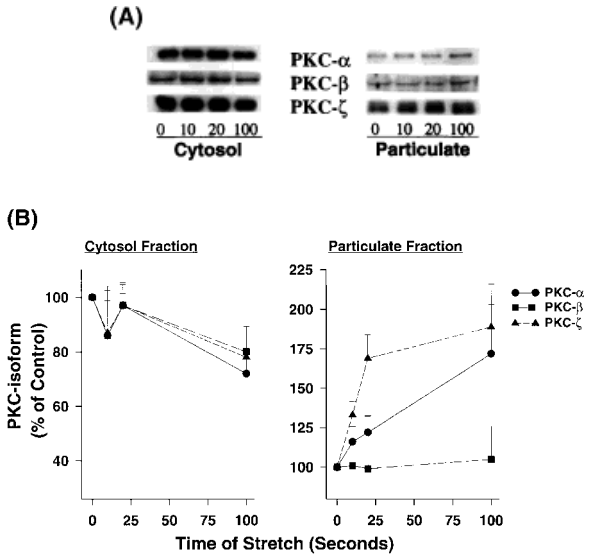

Fig. 2. Effects of cyclic strain on subcellular redistribution of PKC isoforms in SMC. Confluent SMC were subjected to an average 10% strain at 60 cycles/min for up to 100 s. After indicated time cells were harvested and fractionated into cytosol and particulate fraction by differentiated centrifugation. Same amount of proteins were resolved in 7.5% SDS-PAGE and electroblotted to nitrocellulose membrane prior to Western blotting for PKC-α, -β, and -ζ protein. **A**: Modulation of subcellular distribution of PKC isoforms by cyclic strain. Representative blots from three independent experiments. Lane 1 (static control); lane 2 (10 s); lane 3 (20 s); lane 4 (100 s) **B**: Densitometric analysis of immunoblot for PKC isoforms. Data are expressed as % of static control. Results are shown as means ± SE of three independent experiments, $^*P < 0.05$.

1997]. While their specific functions in SMC have not been well elucidated, each isoform is expected to have some unique roles. For example, the response to various stimuli is different between classical PKC (cPKC)-α or -β and atypical PKC (aPKC)-ζ [Khalil et al., 1992; Grange et al., 1998; Inoguchi et al., 1992]. Furthermore, over-expression of PKC-α stimulates cell differentiation [Haller et al., 1995] and lipopolysaccharide-induced nitric oxide formation [Li et al., 1998] in SMC. PKC-δ was also reported to inhibit proliferation of SMC [Fukumoto, 1997]. In addition, consistent with properties of classical PKC isoforms, short exposure of SMC to PMA caused an increase of membrane fraction of PKC-α and -β (βII) [Inoguchi, 1994]. However, only PKC-β isoform was activated in SMC by glucose treatment, which increases DAG level. PKC-ζ isoform was also translocated to membrane fraction in SMC by phenylephrine treatment, which causes smooth muscle contractile [Khalil et al., 1992].

Since our previous studies strongly suggest that cyclic strain modulate cellular signal through the Ca^{2+}-dependent mechanism, we examined the redistribution of cPKC, PKC-α, and -β by cyclic strain. These isoforms were redistributed in bovine endothelial cells (EC) by cyclic strain [Rosales and Sumpio, 1992]. In addition, recent studies have reported that Ca^{2+}-independent and phorbol ester-insensitive PKC isoforms could be involved in many cellular signals. Therefore, we also examined if aPKC are involved in mechanical strain-mediated cellular signals by examining the distribution of PKC-ζ, the most prevalent aPKC isoform in bovine aortic SMC. We found that cyclic strain rapidly redistributed PKC-α and -ζ isoforms. It is interesting to note that the cyclic strain-mediated activation of PKC isoforms might be tissue specific since cyclic strain translocates PKC isoforms in human intestinal cells and keratinocytes in a different manner. While cyclic strain translocates both PKC-α and -ζ isoforms in human intestinal Caco-2 cells [Han et al., 1998] as shown in SMC, cyclic strain only translocates PKC-α but not -ζ isoform in human keratinocytes [Takei et al., 1997]. The increase of particulate PKC isoforms in the present study is consistent with our previous results that specific PKC activity in the membranous pool are increased in SMC by cyclic strain [Mills et al., 1997]. Only a small change in the cytosol pool is expected in PKC-α and ζ, since $< 70\%$ of these PKC isoforms are found to be located in the cytosol. Therefore, a small change in the cytosol would be magnified in the particulate fraction. Particulate translocation of PKC from cytosol has been extensively used as measure of its activation and such translocation occurs in response to agonist-stimulated diacylglycerol synthesis, and this event can be mimicked by phorbol ester and cell permeate diacylglycerol analogues [Nishizuka, 1995, 1998]. However, the finding that PKC-ζ translocates to the membrane following exposure to cyclic strain provides evidence for its activation.

Two potential upstream mechanisms for the activation of PKC in response to cyclic strain may be proposed based on previous studies by our and other laboratories. First, phospholipase C is activated by cyclic strain, resulting in the cleavage of phosphatidylinositol bisphosphate and generation of inositol 1,4,5-triphosphate and diacylglycerol as our laboratory

previously reported [Rosales and Sumpio, 1992]. PKC-α and -β are cPKC isoforms that are activated by diacylglycerol. Thus one mechanism for activation of PKC isoforms is through cyclic strain-mediated generation of diacylglycerol. In many cells, activation of a phosphatidylinositol-specific phospholipase C hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) to produce the intracellular second messengers inositol-1,4,5-triphosphate (InsP3) and diacylglycerol. Once formed, InsP3 causes mobilization of Ca^{2+} from intracellular stores while diacylglycerol activates PKC, an effect mimicked by phorbol esters. Second, the activator of PKC-ζ, aPKC isoform, such as phosphatidylinositol 3,4,5-triphosphate, may be increased in response to cyclic strain. Reports show that these phosphoinositides generated by PI 3-kinase activity are potent activator of PKC-ζ isoform [Herrera-Velit et al., 1997]. It has suggested that PI-3 kinase can activate PKC-ζ in a cell specific and stimulus specific manner. Furthermore, it has been shown that PKC-ζ activity was abrogated by wortmannin, PI3 kinase inhibitor. Therefore, it will be interesting to examine the changes of PI3 kinase activity in response to cyclic strain and how PKC-ζ maintain its activation following exposure to PIP3. The mechanisms leading to the activation of PKC-ζ in cells in response to external stimuli are not fully understood.

Different levels of expression of various isoforms have been correlated with cellular growth and differentiation, and functions [Dekker and Parker, 1994; Hug and Sarre, 1993; Clemens et al., 1992]. Furthermore, the subcellular redistribution of a specific isoform has been matched to a specific cellular event. PKC-α activation is implicated in arachidonate release from MadinDarby canine kidney cells stimulated with bradykinin [Godson, 1990], in the antiproliferative effect of interferon-α on HeLa cells [Pfeffer et al., 1990]. PKC-α translocates in nuclear envelope in NIH 3T3 [Leach et al., 1989], mediating PKC-induced changes in gene expression. PKC-ζ is known to be critically important for regulating a number of cellular functions, including proliferation and activation of nuclear factor, NF-κB [Devary et al., 1993; Dominguez et al., 1993]. It is interesting to note that NF-κB has been found to be activated in EC exposed to strain. Previous studies indicated that PKC-ζ acts downstream of Ras in the kinase cascade leading to mitogenesis [Berra et al., 1995; Ueda et al., 1996]. Stimulation of Ras initiates a kinase cascade that culminates in the activation of MAP kinase, which is required for cell growth and other cellular responses. However, the roles of isoforms in specific cells have yet to be determined. Because each isoform is expressed in a cell (tissue)-dependent manner, it is expected that each isoform plays some unique roles [Dekker and Parker, 1994; Hug and Sarre, 1993; Buchner, 1995].

In the present study, to elucidate the possible mechanisms of an activation of PKC in cyclic strain-induced signal transduction cascade in SMC, we investigated the isoform distribution of PKC in SMC. Except PKC-β isoform, both PKC-α and -ζ isoforms were predominantly located in the cytosol. As the activation of PKC is reflected by its translocation from the cytosol to the membrane fraction [Nishizuka, 1995], these findings suggest that cyclic strain-mediated hydrolysis of PIP2 and PIP3 might result in activation of PKC-α and -ζ, respectively, in SMC. The cyclic strain-mediated activation of these PKC isoforms may initiate a cascade of function and proliferation in response to mechanical force in SMC by stimulating transcription factors, such as NF-κB. Further investigations are required to delineate specific mechanisms of cyclic strain-mediated translocation of PKC isoforms on cellular functions and proliferation in SMC.

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