Platelet-Derived Growth Factor and Angiotensin II Induce Different Spatial Distribution of Protein Kinase C-α and -β in Vascular Smooth Muscle Cells

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Abstract

Protein kinase C is an important second-messenger system that is translocated from the cytosol to the cell membrane on cell stimulation. We used confocal microscopy to study the spatial distribution of protein kinase C isoforms after stimulation of cultured vascular smooth muscle cells with platelet-derived growth factor and angiotensin II (Ang II). Monoclonal antibodies for the isoforms α and β were used. Translocation was also assessed by Western blot. Isoform α was evenly distributed in the cytosol, whereas the β isoform formed coarse granules in the perinuclear region. Both isoforms shifted from the cytosolic to the membrane fraction after exposure to Ang II (10⁻⁷ mol/L) and platelet-derived growth factor (100 ng/mL at 6, 12, and 20 minutes). Confocal microscopy showed a rapid assembly of isoform α along cytosolic fibers at 6 minutes followed by a translocation toward the nucleus at 12 minutes with Ang II. Platelet-derived growth factor engendered a similar response; however, a cytoskeletal distribution was not observed. The β isoform was rapidly translocated by both inducers to the perinuclear region and the nucleus. Our results show that inducers cause a translocation of protein kinase C isoforms not only into the cell membrane but also into the cell nucleus. We suggest that protein kinase C may also be important for nuclear signaling.

Key Words • protein kinase C • muscle, smooth, vascular • microscopy • angiotensin II • platelet-derived growth factor

Protein kinase C (PKC) is a group of calcium- and phospholipid-dependent protein kinases (isoforms) involved in signal transduction responses. Activation of PKC has been implicated in vascular smooth muscle cell (VSMC) contraction, DNA synthesis, and cell growth (for review see Reference 7). PKC is located in the cytosol of VSMCs and other cells during the resting state but is "translocated" to the cell membrane after stimulation. However, because the membrane fraction includes not only cell membranes but also nuclear material, earlier studies do not allow a precise definition of subcellular PKC distribution after cell stimulation. Other investigators observed that only a portion of PKC is shifted to the surface membranes and that PKC is associated with nuclear membranes and cytoskeletal proteins, which raises the possibility that PKC may also perform important tasks elsewhere within the cell.

The pattern of isoform distribution may also vary depending on the activation state of the cell or the agonists used. In VSMCs the calcium-sensitive PKC isoforms α and β as well as the calcium-independent PKC isoforms ε and ζ have been described. We tested the hypothesis that PKC isoforms have different distributions in VSMCs at rest and during agonist stimulation. We used confocal microscopy to examine the effects of two different agonists, angiotensin II (Ang II) and platelet-derived growth factor (PDGF), on the spatial distribution of PKC isozymes α and β in intact VSMCs. We compared this method with the translocation observed after cell fractionation and Western blot.

Methods

Preparation of VSMCs

Rat aortic VSMCs were cultured by procedures modified from Kariya et al. Male Wistar-Kyoto rats (12 to 14 weeks) were anesthetized and bled, and their thoracic aortas were excised. After adherent fat and connective tissue were removed, the aorta was cut longitudinally, and the endothelial cells were removed by gentle scraping with fine forceps. The aortas were then minced into small pieces and incubated at 37°C for 2 hours in phosphate-buffered saline (PBS) without calcium but with 1 mg/mL collagenase (type I, 150 IU/mg, Worthington Biochemical Corp), 0.5 mg/mL elastase (type III, 40 IU/mg, Sigma Chemical Co), and 0.5 mg/mL trypsin inhibitor (Sigma). After 2 hours Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (GIBCO) was added to the suspension to inactivate enzymes. The cells were then centrifuged at 120g for 10 minutes, and the pellet was resuspended in DMEM with 10% fetal calf serum. Then cells were seeded at a density of 3 × 10⁴/cm² and cultured at 37°C in 95% air plus 5% CO₂. Cells from passages 2 through 4 were used in all experiments. The phenotype of the cultured VSMCs was determined by staining the cells for α-actin and desmin. Ang II was obtained from Sigma, and PDGF-AB was purchased from Boehringer Mannheim.

Western Blot

After the experiments the cultured VSMCs were treated with ice-cold homogenization buffer (mmol/L): Tris-HCl 20, pH 7.5, sucrose 250, EGTA 3, mercaptoethanol 10, phenyl-

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methylsulfonyl fluoride 1, and leupeptin 50) and were immediately homogenized. The homogenate was then spun at 100 000g for 60 minutes, and the supernatant was used as the cytosolic fraction. The pellet was resuspended in buffer containing 1.0% Triton X-100 and centrifuged at 100 000g for another 60 minutes. The supernatant was used as the PKC-containing fraction of the original membrane pellet. Both PKC-containing fractions then underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% SDS gels. Protein (50 μg) was loaded onto each lane. The fractions were then electroblotted onto Immobilon-P membranes (Millipore). The membranes were successively incubated, first with blocking buffer containing 137 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 3% (wt/vol) bovine serum albumin (BSA), 0.2% (vol/vol) Tween 20, and 0.02% NaN₃, for 120 minutes at room temperature. The next incubation was conducted in affinity-purified, isoenzyme-specific antibody diluted in incubation buffer containing 137 mmol/L NaCl and 20 mmol/L Tris-HCl (pH 7.5) at room temperature. We used highly specific monoclonal antibodies directed against rabbit brain PKC that reacted specifically with the α (MC-3a), β (MC-2a), and γ (MC-1a) subtypes of PKC (Seikagaku Kogyo Ltd). A final incubation was carried out in triethanolamine-buffered saline with biotinylated anti-rabbit IgG (Amersham) and streptavidin-alkaline phosphatase peroxidase (Calbiochem) complex in the incubation buffer. The membranes were thoroughly washed after each incubation with a buffer containing 137 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), and 0.2% (vol/vol) Tween 20. Quantification of the 80-kd PKC band was done by densitometry on a video densitometer 620 (Bio-Rad Laboratories). The signals were then integrated and the results expressed in arbitrary units.

**Immunocytochemistry**

VSMCs were fixed with 3% paraformaldehyde and permeabilized with ice-cold 80% methanol. After incubation with 3% skimmed milk in PBS for 60 minutes, the preparation was incubated for 1 hour at room temperature with the PKC antibodies (GIBCO) diluted in 0.1% BSA (1:80), washed three times with PBS, and then exposed to the secondary antibody (fluorescein isothiocyanate–conjugated anti-mouse IgG, at 1:100, 0.1% BSA/PBS [Pierce Chemicals]) for 60 minutes. The preparation was mounted with 50% glycerol under a glass coverslip on a Nikon Diaphot microscope. A Bio-Rad MRC 500 confocal imaging system with an argon laser was used. At least 10 to 18 cells from each of at least five experiments were examined under each experimental condition. The results were reproduced by two separate investigators, and multiple experiments were done. The observers were unaware of the experimental design and antibodies used.

Quantification of the signal intensity in nuclear, perinuclear, cytoplasmic, and periplasmic membrane regions was done with AREA functions in the MRC software. The subcellular regions were outlined manually, and the calculated mean fluorescent intensity was obtained for the delineated regions. Data are presented as the ratio of the mean fluorescent intensity in the respective regions to the mean fluorescent intensity of the whole cell area.

Statistical analysis was carried out on a Macintosh II computer (Apple Inc) with a commercial program (STATVIEW, Cricket Software Inc). The results are expressed as mean ±SEM. The nonparametric Wilcoxon test was used. Differences were considered to be significant when the probability value was less than or equal to .05.

**Results**

Fig 1, a representative example of three experiments, shows a Western blot of an 89-kD protein representing PKC-α and PKC-β. We invariably observed a faint band at 40 kD, which most likely represented breakdown products of PKC. No other proteins were present. In resting VSMCs both PKC-α and PKC-β isoforms were present within the cytosol, whereas PKC-γ was not detectable. The expression of PKC-β was lower than that of PKC-α. After cell fractionation both isoforms were located primarily in the cytosol; almost no immunoreactivity was observed in the particulate fraction.

**Fig 2** is a confocal photomicrograph showing the immunofluorescence of PKC-α and -β. The isoforms showed different staining patterns (Fig 2A and 2C). PKC-α showed a diffuse cytosolic pattern with a homogeneous distribution. PKC-β on the other hand demonstrated a more coarse pattern, with increased staining in the perinuclear region (Fig 2C). In resting VSMCs no PKC immunoreactivity in the perimembranous region was observed. In a few cells a slight nuclear staining for both PKC-α and -β was found.

When Ang II (10⁻⁷ mol/L) was added to VSMCs, the subcellular distribution of PKC-α changed rapidly. In the fractionation experiments Ang II induced a significant translocation of PKC-α from the cytosolic compartment to the particulate fraction (Fig 1). Using confocal microscopy we observed a significant increase in PKC-α immunoreactivity in the perinuclear region at 6 minutes. There were also distinct focal points of PKC-α immunoreactivity in the cell membrane. In the cytosolic region PKC-α immunoreactivity changed from a homogeneous distribution to a linear pattern. After 12 minutes this linear pattern was more intense, suggesting an association of PKC-α with cytoskeletal proteins. The focal points in the perimembranous region were virtually unchanged. On the other hand, the PKC-α immunoreactivity in the perinuclear region was significantly diminished. The PKC immunoreactivity in the nucleus showed an intense staining. Total immunoreactivity of PKC in stimulated cells appeared to be increased compared with resting cells.

**Fig 3A** shows the percent changes in immunofluorescence of PKC-α in the nuclear, perinuclear, and perimembranous regions (mean values of six experiments). Ang II induced a modest translocation of PKC-α to the plasma membrane, whereas the bulk of the enzyme was shifted toward the perinuclear region and into the nucleus.

When we examined the effect of Ang II on PKC-β, we also observed a translocation of PKC immunoreactivity from the cytosol to the particulate fraction (Fig 1). However, the effect of Ang II on the subcellular distribution of PKC-β was different from that of PKC-α.
PKC-β was concentrated in patchy spots inside the nucleus. Almost no PKC-β was translocated toward the perimembranous region. No fibrillar organization of PKC-β in the cytosolic region was noted. After 12 seconds most of the PKC-β immunoreactivity was located in the perinuclear region, with “hot spots” inside the nucleus (Figs 2C and 3B).

PDGF-AB (100 nmol/L) also induced a translocation of both PKC isozymes from the cytosolic to the particulate fraction (Fig 1). PDGF caused only a small shift of PKC-α to the perinuclear region (6 minutes), followed by an increase in nuclear PKC-α immunoreactivity at 12 minutes (Fig 2B). PKC-α immunoreactivity induced by PDGF was less than that induced by Ang II (Fig 2A and 2B). The pattern of nuclear staining after PDGF was also different from Ang II; PDGF induced no homogeneous distribution but rather a patchy heterogeneous staining inside the nucleus. With PDGF the characteristic cytosolic fibrillar pattern induced by Ang II was not observed. Although PDGF showed different effects on the subcellular distribution of PKC-α compared with Ang II, both hormones had similar effects on PKC-β. PDGF increased the nuclear immunoreactivity of PKC-β in hot spots, with a concomitant increase in perinuclear PKC-β immunoreactivity.

Discussion

Our main findings are the different effects of Ang II and PDGF on the subcellular translocation of PKC isozymes in VSMCs. We also demonstrated that the translocation of PKC immunoreactivity from the cytosol involves not only movement to the cell membrane but also a shift of PKC toward the perinuclear region and into the nucleus. PKC isoforms have been demonstrated in VSMCs by other researchers.9'18 The observation that PKC-β expression is weaker than that of PKC-α confirms earlier observations from our group and others.19'20 Both Ang II and PDGF induced a similar translocation of PKC isozymes from the cytosolic fraction to the particulate fraction, which has been demonstrated previously.8'21'22 However, only Ang II induced the translocation of a small amount of PKC-α to the plasma mem-
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may perform separate functions in the nucleus. Control. The different intranuclear distribution of PKC-α and -β in our study suggests that the isozymes previously recognizes a domain that is more accessible in the activated enzyme (personal communication from the objective determination of intensity. We believe that the increase in PKC immunoreactivity is due to the unfold-

Fig 3. Bar graphs show quantitation of relative distribution of protein kinase C-α associated with the perimembrane, perinuclear, and nuclear regions after exposure of vascular smooth muscle cells to angiotensin II (10^{-8} mol/L) (A) or platelet-derived growth factor (100 ng/mL) (B) for 6 and 12 minutes. *P<05 compared with control values.

brane. Most PKC immunoreactivity was shifted toward the nucleus. The translocation of PKC-α into the cell nucleus has recently been observed by other investigators in several cell types. However, in those experiments phorbol esters were used as activators of PKC. Our results are the first to demonstrate that physiological activators of PKC in VSMCs lead to the translocation of these isozymes to the nucleus. The time course of the changes in PKC immunoreactivity suggests that PKC-α and -β are first shifted toward the perinuclear region and then translocated into the nucleus. The mechanism for this translocation is not clear, but it has been suggested that PKC cycles into the nucleus and is “fixed” by locally generated diacylglycerol.

In stimulated cells PKC immunoreactivity was not only translocated into the nucleus but also increased in intensity. The increase in intensity is unlikely to be due to a two-dimensional representation of a three-dimensional phenomenon, because the confocal microscope permits the visualization of a single layer or vertical dimension through the cell. This feature allows a more objective determination of intensity. We believe that the increase in PKC immunoreactivity is due to the unfolding of the enzyme after activation. The antibody obviously recognizes a domain that is more accessible in the activated enzyme (personal communication from the manufacturer). This phenomenon has also been observed in other investigations.

Several reports have suggested that PKC has intranuclear functions, and our findings support this notion for PKC-α and -β in VSMCs. Possible PKC substrates in the nucleus include laminas, DNA polymerase, and topoisomerase II (for review see Reference 27). PKC isozymes may therefore play a role in transcriptional control. The different intranuclear distribution of PKC-α and -β in our study suggests that the isozymes may perform separate functions in the nucleus.

Ang II induced an association of PKC-α with cytoskeletal structures, whereas PDGF did not. Khalil and Morgan used a fluorescent probe for total PKC and investigated PKC translocation in isolated cells in response to phenylephrine. They reported a translocation to the surface membrane and a diffuse staining in the cytosol and perinuclear region. In contrast, Mochly-Rosen et al observed a translocation of a PKC isozyme to cytoskeletal structures in neonatal cardiac cells after exposure to phorbol ester. Leach et al described an association of PKC-α with cytoskeletal structures in fibroblasts. The PKC-α pattern in their study is very similar to our findings. They suggested a colocalization of PKC-α with the cytoskeletal protein talin, an observation we confirmed in preliminary experiments (unpublished observation). Although both Ang II and PDGF induced activation of PKC, they caused a different subcellular distribution of PKC. The lack of translocation to cytoskeletal structures by PDGF suggests that Ang II-specific intracellular signals are responsible. Ang II and PDGF share inositol 1,4,5-trisphosphate generation and phospholipase C-dependent diacylglycerol generation, so the specific effect of Ang II on PKC-α may be due to Ang II-dependent activation of G proteins, phospholipase D, or both.

We suggest that the translocation of PKC-α and -β into the nucleus indicates that these isoenzymes may play a role in the early growth response to Ang II and PDGF. Further experiments will be necessary to investigate the role of PKC isozymes in the nucleus and to understand the mechanisms responsible for the differential cytoskeletal distribution of PKC isozymes induced by growth-promoting factors.

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References


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