Acetylcholine Activates Protein Kinase C-α in Pulmonary Venous Smooth Muscle

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Background: The authors investigated whether acetylcholine-induced contraction in pulmonary venous smooth muscle (PVSM) is associated with the activation of specific protein kinase C (PKC) isoforms.

Methods: Isolated canine pulmonary venous rings without endothelium were suspended in modified Krebs-Ringer's buffer for measurement of isometric tension. The effects of nonspecific PKC inhibition (bisindolylmaleimide I; 3×10^{-6} M) and conventional PKC isoform inhibition (Gö7936 10^{-6} M) on the acetylcholine dose–response relation were assessed. The expression of conventional PKC isoforms (α, β, γ), novel PKC isoforms (δ, ε, θ), and atypical PKC isoforms (ζ, ι, μ) was measured in PVSM cells by Western blot analysis. The immunofluorescence technique and confocal microscopy were used to localize the cellular distribution of PKC isoforms before and after the addition of acetylcholine.

Results: Acetylcholine caused dose-dependent contraction in E-pulmonary veins. Pretreatment with bisindolylmaleimide I or Gö7936 attenuated acetylcholine contraction. PKC-α, -ι, -μ, and -ζ were expressed, whereas PKC-β, -γ, -δ, -ε, and -θ were not expressed in PVSM cells. Immunofluorescence staining for PKC isoforms showed that in unstimulated cells, PKC-α and PKC-μ were detected only in the cytoplasm. PKC-ι and PKC-ζ also exhibited a cytoplasmic immunofluorescence pattern, which was especially abundant in the perinuclear zone. Activation with acetylcholine induced translocation of PKC-α from cytoplasm to membrane, whereas acetylcholine had no effect on the other PKC isoforms. Translocation of PKC-α in response to acetylcholine was blocked by the muscarinic receptor antagonist, atropine.

Conclusion: Acetylcholine contraction is attenuated by PKC inhibition in PVSM. Acetylcholine induces translocation of PKC-α from cytoplasm to membrane in PVSM. These results suggest that PKC-dependent acetylcholine contraction in PVSM may involve activation and translocation of PKC-α.

PULMONARY veins (PVs) are a primary site for entry of vagal nerves into the left atrium.[1] Pulmonary venous constriction may be involved in pulmonary edema formation in congestive heart failure,[2] as well as in high-altitude pulmonary edema.[3] We have previously demonstrated that the muscarinic receptor agonist, acetylcholine, caused contraction in PVs,[4] which is mediated by Ca^{2+} influx, Ca^{2+} release, and an increase in myofilament Ca^{2+} sensitivity. Moreover, the protein ki-

nase C (PKC), rho kinase, and tyrosine kinase signaling pathways each contributed to the acetylcholine-induced increase in myofilament Ca^{2+} sensitivity in PVs.[4] We have also demonstrated that the intravenous anesthetic, ketamine, attenuated acetylcholine contraction *via* the PKC signaling pathway.[5]

Protein kinase C enzymes can be divided into three classes: conventional, novel, and atypical.[6,7] The conventional PKCs are represented by α, β, and γ isoforms that are activated in a Ca^{2+}-dependent manner in the presence of phosphatidylserine. These isoforms also bind to diacylglycerol or phorbol esters such as phorbol 12-myristate 13-acetate (PMA), which increases the specificity of the enzymes for phosphatidylserine and shifts their affinity for Ca^{2+} to the physiologic range. The novel class of PKC isoforms consists of δ, ε, θ, and η isotypes. These kinases are Ca^{2+} insensitive but are activated by diacylglycerol or PMA. The atypical PKC isoforms are comprised of ζ, ι/λ, and μ isotypes that are Ca^{2+} insensitive and are not activated by diacylglycerol or PMA. Although PKC has been reported to mediate excitation–contraction coupling in a wide variety of excitable cells,[8–10] the roles of specific isoforms in mediating this function in pulmonary venous smooth muscle (PVSM) are not known. In the current study, we tested the hypothesis that specific PKC isoforms play an essential role in the contractile response to acetylcholine in PVSM. This study is a prerequisite to elucidate the extent and the cellular mechanism of action by which anesthetic agents may alter the pulmonary vascular responses to vasoconstrictor stimuli.

Materials and Methods

Animals

Pulmonary veins were isolated from adult mongrel dogs. The technique of euthanasia was approved by the Cleveland Clinic Institutional Animal Care and Use Committee, Cleveland, Ohio. All steps were performed aseptically during general anesthesia with intravenous pentobarbital sodium (30 mg/kg) and intravenous fentanyl citrate (20 μg/kg). The dogs were intubated and ventilated. After the administration of heparin (6,000 U), the dogs were exsanguinated by controlled hemorrhage *via* a femoral artery catheter and killed with electrically induced ventricular fibrillation. A left lateral thoracotomy was performed, and the heart and lungs were removed *en bloc*. The pulmonary veins were isolated and dissected in the laboratory for organ chamber experiments or in a laminar flow hood using sterile procedures for cell culture.

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Preparation of Pulmonary Venous Rings

Intralobar PVs (third generation, 1–2 mm ID) were carefully dissected and immersed in cold modified Krebs-Ringer's bicarbonate solution composed of 118.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $NaHCO_3$, 0.016 mM Ca-EDTA, and 11.1 mM glucose. PVs were cleaned of connective tissue and cut into ring segments 4–5 mm in length. The endothelium was removed by gently rubbing the intimal surface with a cotton swab. The integrity of the endothelium was verified by assessing the vasorelaxant response to the endothelium-dependent vasodilator, bradykinin (10^{-8} M),[11] during acetylcholine contraction. Bradykinin induced more than 20% relaxation in endothelium-intact PV rings, and no relaxation or a slight contraction in endothelium-denuded PV rings.[4,10]

Isometric Tension Experiments

Pulmonary vein rings were vertically mounted between two stainless steel hooks in organ baths filled with 25 ml Krebs-Ringer's bicarbonate solution (37°C) gassed with 95% O_2 and 5% CO_2. One of the hooks was anchored, and the other was connected to a strain gauge to measure isometric force. The rings were stretched at 5-min intervals in increments of 0.5 g to achieve optimal resting tension. Optimal resting tension was defined as the minimal amount of stretch required to achieve the largest contractile response to 60 mM KCl and was determined in preliminary experiments to be 1.5 g. After the PV rings had been stretched to their optimal resting tension, the contractile response to 60 mM KCl was assessed. After washout of KCl from the organ chamber and the return of isometric tension to prestimulation values (i.e., no precontraction), a concentration–response curve to acetylcholine was performed in each ring. This was achieved by increasing the concentration of acetylcholine in half-log increments (from 0.01 to 10 μM) after the response to each preceding concentration had reached a steady state. To assess the role of the PKC signaling pathway in the contractile response to acetylcholine, E-PV rings were pretreated for 30 min with bisindolylmaleimide I (BIS1, 3 μM), a nonspecific PKC inhibitor, or a conventional PKC isoform inhibitor, Gö6976 (1 μM). The contractile responses to acetylcholine in BIS1- or Gö6976-pretreated rings were compared with responses in untreated paired rings. We have previously used 3 μM BIS1 to inhibit the PKC signaling pathway in PVs.[4,5,10] Gö6976 (1 μM) has been previously used to inhibit conventional PKC isoforms.[12,13]

Cell Culture of PVSMCs

Primary cultures of pulmonary venous smooth muscle cells (PVSMCs) were obtained from segmental and subsegmental branches of PVs (the third and fourth generation having diameters < 4 mm). The intralobar veins were carefully dissected and prepared for tissue culture.

Explant cultures were prepared according to the method of Campbell and Campbell,[14] with minor modifications. Briefly, the endothelium was removed by gently rubbing with a sterile cotton swab. The tunica adventitia was carefully removed, together with the most superficial part of the tunica media. The remaining portion of the media was cut into 1-mm² pieces that were explanted on precleaned 22-mm² glass coverslips placed individually in six-well culture plates for immunofluorescence studies, or into 25-cm² culture flasks to generate large numbers of cells for Western blot analysis. The explants were nourished with Dulbecco's modified Eagle's medium–F-12 containing 10% fetal bovine serum and 1% antibiotic mixture solution (10,000 U/ml penicillin and 10,000 μg/ml streptomycin) and kept in a humidified atmosphere of 5% CO_2–95% air at 37°C. PVSMCs began to proliferate from explants after 7 days in culture. Cells were allowed to grow for an additional 10–14 days until subconfluence was achieved. The cells were never passed. The cells exhibited morphologic characteristics of vascular smooth muscle as confirmed by α-actin stain, and expressed α-actin as assessed by Western blot analysis.

Western Blot Analysis

Confluent PVSMCs grown from explants were washed with PBS solution. Protein was extracted by M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Inc. Rockford, IL). Gel electrophoresis was performed with 10% polyacrylamide gels to separate the solubilized PVSMC protein. In addition, positive controls for all of the isoform-specific antibodies were performed with rat cerebrum lysate (α, β, γ, ε, and δ), K-562 whole cell lysate (μ), Hela whole cell lysate (ι/λ and ζ), or Jurkat cell lysate (θ). After transfer of the proteins from the gel to a nitrocellulose membrane, the remaining protein binding sites were blocked with 5% nonfat dry milk. The membranes were subsequently incubated with the monoclonal isoform-specific PKC antibody (α, β, γ, ε, δ, and ι) or polyclonal PKC antibody (μ, ζ, and θ) overnight at 4°C. The nitrocellulose membranes were washed in Tris-buffered saline–Tween-20 (0.1%) (three times) and incubated with anti-mouse or anti-rabbit conjugated horseradish peroxidase for 1 h at room temperature. After several washes in Tris-buffered saline (3 × 30 min), the membranes were developed with enhanced chemiluminescence solution for 1 min and exposed to film.

Immunofluorescence Labeling of PKC Isoforms

Primary cultures of PVSMCs were divided into five experimental groups. All groups were rendered quiescent with medium lacking fetal bovine serum for 24 h before experimentation. The first group of cells was untreated. The second group was treated with acetylcholine (10^{-6} M). This dose was selected because it

caused contraction and increased myofilament Ca^{2+} sensitivity in PVSM.[4] The third group was exposed to the PKC activator, PMA (10^{-6} M), as a positive control. The fourth group was exposed to atropine (10^{-6} M), a muscarinic receptor antagonist. The fifth group was exposed to acetylcholine after pretreatment with atropine. All treatments were for 15 min. The experiments were reproduced in cells from at least four individual dogs on 3 separate days. The indirect immunofluorescence technique was used to localize the cellular distribution of PKC isoforms. Immediately after each treatment, the reaction was stopped by placing the coverslips in 1:1 (vol/vol) acetone–methanol at 20°C for 10 min to simultaneously fix the cells and permeabilize their plasma membranes. Fixed cells were then washed with 0.1 M phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 10 min, and subsequently incubated with isoform-specific PKC antibodies at a dilution of 10 μg/ml in PBS–BSA overnight at 4°C. Monoclonal isoform-specific PKC antibodies (α, β, γ, ε, δ, and ι) or polyclonal PKC antibodies (μ, ζ, and θ) were used for the immunofluorescence protocols. After incubation with the primary antibody, the coverslips were thoroughly washed in PBS–BSA and incubated with fluorescein isothiocyanate–conjugated goat anti-mouse or goat anti-rabbit immunoglobulin G (secondary antibody) diluted 1:400 in PBS–BSA for 60 min at 37°C. An immunocytochemical control for antibody specificity was performed by incubating the cells with the secondary antibody only. After a thorough washing in PBS–BSA, the coverslips were mounted on microscope slides with Aquamount (BDH Laboratory, Dorset, UK). The specimens were viewed and photographed using confocal microscopy. Single optical sections (1,024 × 1,024) were collected with a Leica TCS-SP AOBS laser scanning confocal microscope (Leica-Microsystems, Heidelberg, Germany) using an HCX plan Apo 40× 1.25N.A. oil immersion objective lens. DAPI was excited with a 351-nm argon laser, and DAPI emission was collected between 387 and 510 nm using the built-in spectrophotometer. Alexa-488–conjugated antibodies bound to PKC were excited with the 488-nm line of an argon laser with emitted light collected between 496 and 563 nm. Each image was frame averaged six times to reduce noise.

Materials

Dulbecco's modified Eagle's medium–F-12, the antibiotic–antimycotic mixture, and BSA (fraction V) were from GIBCO (Grand Island, NY). Acetylcholine, atropine, PMA, BIS1, and Gö6976 were from Sigma (St. Louis, MO). PKC-α, PKC-δ, PKC-ζ, and horseradish peroxidase–labeled goat anti-mouse and goat anti-rabbit immunoglobulin G were from Upstate Cell Signaling Solutions (Lake Placid, NY). PKC (β, γ, ε, and δ) and positive control cell lysates were from BD Biosciences (Lexing-

ton, KY). PKC-μ and -θ antibody and their positive control cell lysates were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated–labeled goat anti-mouse and goat anti-rabbit immunoglobulin G were from Molecular Probes (Eugene, OR). PMA, BIS1, and Gö6976 were dissolved in dimethyl sulfoxide and diluted with distilled water. The final concentration of dimethyl sulfoxide in the organ bath was less than 0.1% (vol/vol).

Data Analysis

All data are expressed as mean ± SD. Contractile responses to acetylcholine in the ring studies are expressed as the percentage contraction induced by 60 mM KCl. The acetylcholine contractile responses were compared in matched control and "treated" rings from the same dogs. Two-way analysis of variance for repeated measures followed by contrast analysis and Bonferroni correction was used for comparisons within and between groups. Acetylcholine dose was used as the within-subject factor, and treatment (with or without) was used as the between-subjects factor. All statistical analyses used SPSS for WINDOWS software (version 11.5; SPSS Inc., Chicago, IL). A P value of less than 0.05 was chosen as significant. In all experiments, sample size (n values) equals the number of dogs from which PV rings were taken.

Results

Role of the PKC Signaling Pathway in Acetylcholine Contraction

We tested the hypothesis that acetylcholine contraction in PVs involves the PKC signaling pathway. Nonspecific PKC inhibition (BIS1) or conventional PKC inhibition (Gö6976) had no effect on resting tension in E-PV. However, BIS1 ($P < 0.001$) and Gö6976 ($P = 0.002$) each attenuated acetylcholine contraction (figs. 1A and B, respectively). These results indicate that the PKC signaling pathway mediates a component of acetylcholine contraction in PVs.

Western Blots of PKC Isoforms

Experiments were performed to assess the specificity of the antibodies. Positive controls for all of the isoform-specific antibodies were performed with rat cerebrum lysate (α, β, γ, ε, and δ), K-562 whole cell lysate (μ), Hela whole cell lysate (ι/λ and ζ) or Jurkat cell lysate (α). Figure 2 shows the PKC isoforms detected by Western blot analysis in the PVSMCs. PKC-α, -μ, -ι, and -ζ (PKC-α at approximately 82 kd, PKC-μ at approximately 115 kd, PKC-ι at approximately 74 kd, and PKC-ζ at approximately 72 kd) each showed two prominent bands, one for positive control, the other for PVSMCs, which indicates that PKC-α, -μ, -ι, and -ζ are expressed in PVSMCs.
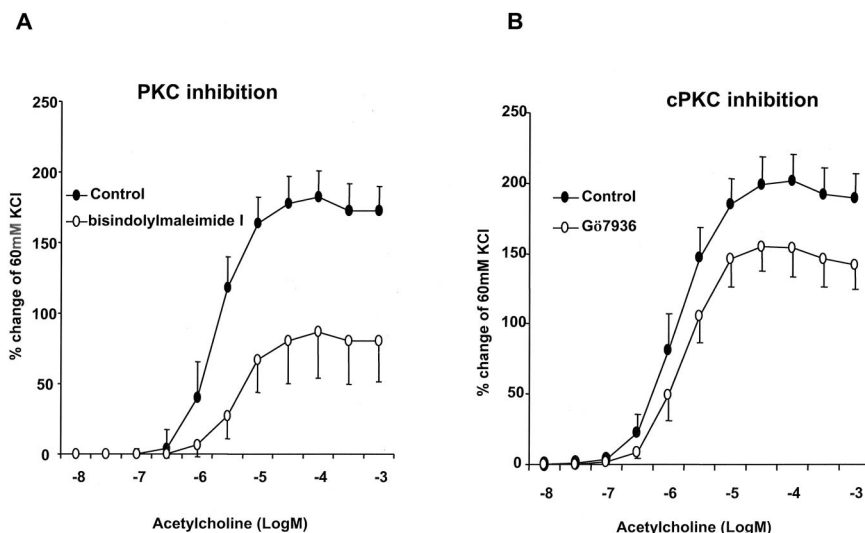
A

B

Fig. 1. (*A*) Effect of protein kinase C (PKC) inhibition (3 μM bisindolylmaleimide I) on acetylcholine contraction in endothelium-denuded pulmonary veins. (*B*) Effect of conventional PKC (cPKC) isozyme inhibition (1 μM Gö7936) on acetylcholine contraction in endothelium-denuded pulmonary veins. Inhibition of PKC ($P < 0.001$) and cPKC ($P = 0.002$) attenuated acetylcholine contraction. n = 6.

In contrast, PKC-β, -γ, -ε, -δ, and -θ blots (PKC-β, -γ, -δ, and -θ at approximately 80 kd and PKC-ε at approximately 90 kd) only showed one prominent band for positive control, which indicates that these PKC isoforms are not expressed in PVSMCs (fig. 3).

Effect of Acetylcholine on the Cellular Distribution of PKC Isoforms

The antibodies that gave a positive result on the Western blot analysis (PKC-α, -μ, -ι, and -ζ) were used for immunocytochemistry. Exposure of PVSMCs to acetylcholine caused translocation of PKC-α from cytoplasm to membrane (fig. 4). However, acetylcholine had no effect on the cellular distribution of the other PKC isoforms (figs. 5–7).

Immunolocalization of PKC-α. In unstimulated cells, PKC-α was detected in the cytoplasm (fig. 4A).

Treatment with PMA caused translocation of PKC-α from cytoplasm to membrane (fig. 4B). Treatment with acetylcholine mimicked PMA-induced translocation of PKC-α to the membrane (fig. 4C). The muscarinic receptor antagonist, atropine, had no effect on the subcellular distribution of PKC-α compared with unstimulated cells (fig. 4D). However, pretreatment with atropine before acetylcholine administration abolished the acetylcholine-induced translocation of PKC-α (fig. 4E).

Immunolocalization of PKC-μ. In unstimulated cells, PKC-μ was detected in the cytoplasm (fig. 5A). In PMA-treated cells, the cytoplasmic staining pattern was similar to that of unstimulated cells (fig. 5B). No redistribution of PKC-μ was observed in cells treated with acetylcholine (fig. 5C).
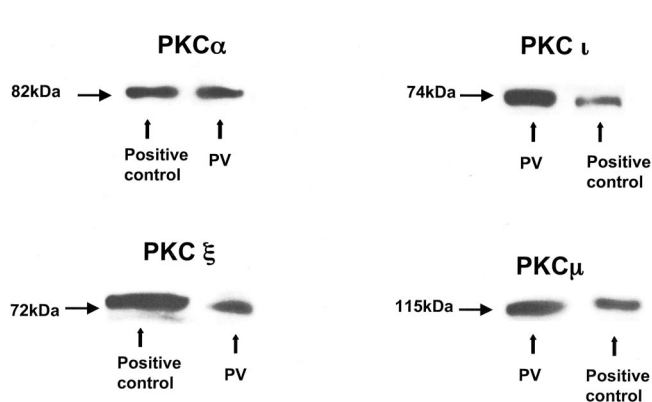

Fig. 2. Expression of protein kinase C (PKC)-α, -ζ, -ι, and -μ in pulmonary venous smooth muscle cells. Western blots for PKC-α, -μ, -ι, and -ζ (PKC-α at approximately 82 kd, PKC-μ at approximately 115 kd, PKC-ι at approximately 74 kd, and PKC-ζ at approximately 72 kd) each showed two prominent bands, one for positive control, the other for pulmonary venous smooth muscle cells, which suggests that PKC-α, -μ, -ι, and -ζ are each expressed in pulmonary venous smooth muscle cells. Positive controls for the isoform-specific antibodies were performed with rat cerebrum lysate (α), K-562 whole cell lysate (μ), and Hela whole cell lysate (ι/λ and ζ). PV = pulmonary vein.
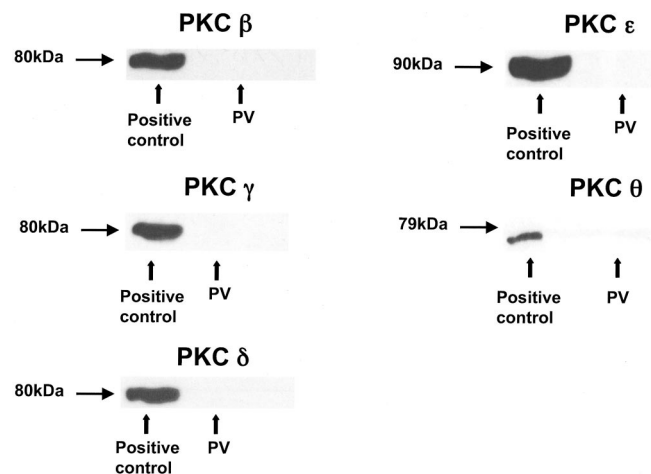

Fig. 3. Expression of protein kinase C (PKC)-β, -ε, -δ, -γ, and -θ in pulmonary venous smooth muscle cells. PKC-β, -ε, -δ, -γ, and -θ blot (PKC-β, -γ, -δ, and -θ at approximately 80 kd and PKC-ε at approximately 90 kd) only showed one prominent band for positive control, which suggests that these PKC isoforms are not expressed in pulmonary venous smooth muscle cells. Positive controls for the isoform-specific antibodies were performed with rat cerebrum lysate (β, γ, ε, and δ) or Jurkat cell lysate (θ). PV = pulmonary vein.

Fig. 4. Immunolocalization of protein kinase C (PKC)-α. In untreated cells, PKC-α was detected in the cytoplasm (A). Treatment with phorbol 12-myristate 13-acetate caused translocation of PKC-α from cytoplasm to membrane (B). Treatment with acetylcholine also induced translocation of PKC-α to membrane (C). The muscarinic receptor antagonist, atropine, alone had no effect on the subcellular distribution of PKC-α compared with unstimulated cells (D). However, pretreatment of pulmonary venous smooth muscle cells with atropine before acetylcholine treatment abolished the acetylcholine-induced translocation of PKC-α (E).
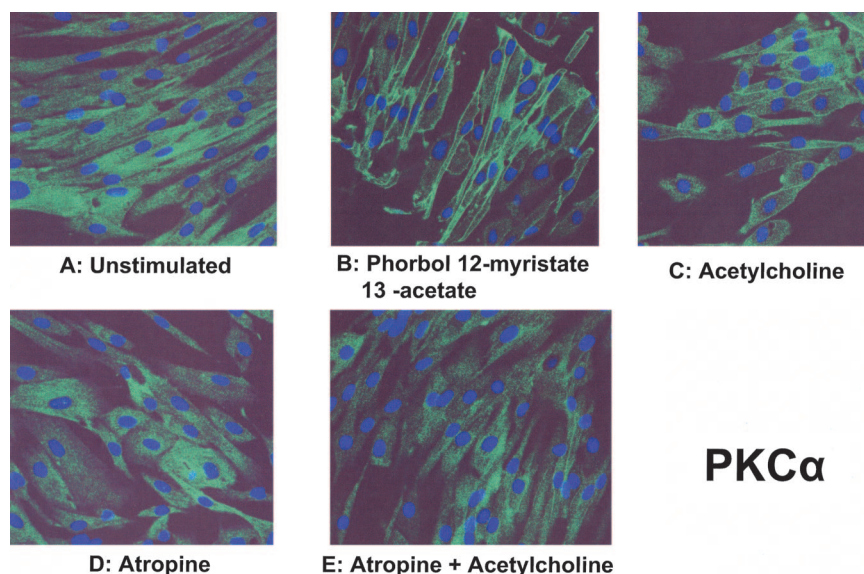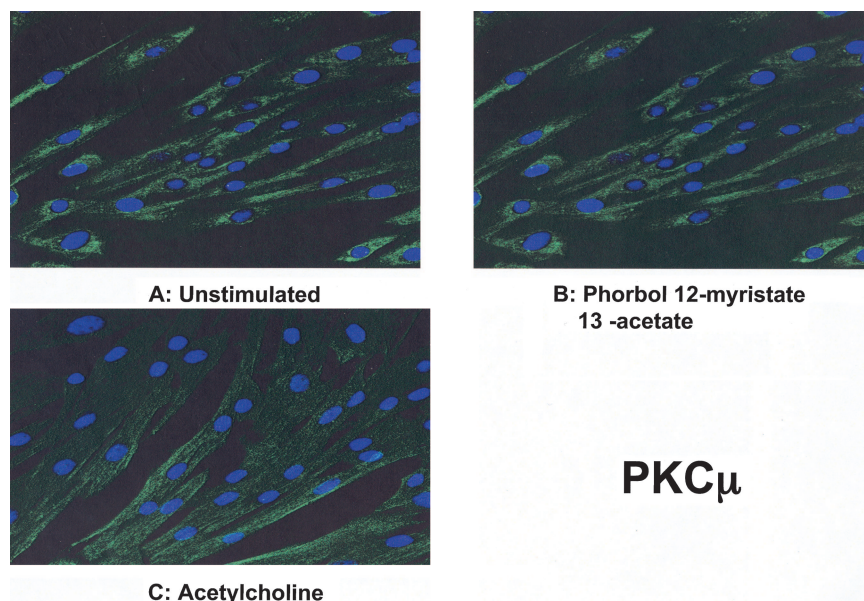
Immunolocalization of PKC-ι. In unstimulated cells, PKC-ι exhibited a cytoplasmic fluorescent pattern, which was especially abundant in the perinuclear zones (fig. 6A). In PMA-treated cells, the cytoplasmic staining pattern was similar to that of unstimulated cells (fig. 6B). No redistribution of PKC-ι was observed in cells exposed to acetylcholine (fig. 6C).

Immunolocalization of PKC-ζ. In unstimulated cells, PKC-ζ exhibited a bright granular cytoplasmic fluorescent pattern, which was especially abundant in the perinuclear zones (fig. 7A). In PMA-treated cells, the cytoplasmic staining pattern was similar to that of unstimulated cells (fig. 7B). No redistribution of PKC-ζ was observed in cells treated with acetylcholine (fig. 7C).

Discussion

Pulmonary veins return oxygenated blood from the lungs to the left heart. PV contraction increases pulmonary capillary pressure, which could result in pulmonary hypertension and pulmonary edema.[15] It has been reported that PV contraction due to hypoxia or thromboxane contributes significantly to pulmonary hypertension and edema formation.[16,17] Increased PV pressure accompanying ventricular failure causes transudation of fluid into the pulmonary capillary interstitium, which limits the transfer of oxygen from alveoli into blood. The resulting hypoxia can lead to further deterioration of ventricular performance and to further decreases in body tissue oxygenation, which results in left ventricular dysfunction and pulmonary edema.[18] PKC plays an important role in the signal transduction pathway mediating

Fig. 5. Immunolocalization of protein kinase C (PKC)-μ. In unstimulated cells, PKC-μ was detected in the cytoplasm (A). No redistribution of PKC-μ was observed in cells exposed to phorbol 12-myristate 13-acetate (B). In acetylcholine-treated cells, the cytoplasmic staining pattern was similar to that of unstimulated cells (C).

A: Unstimulated

B: Phorbol 12-myristate 13 -acetate

C: Acetylcholine

PKCι
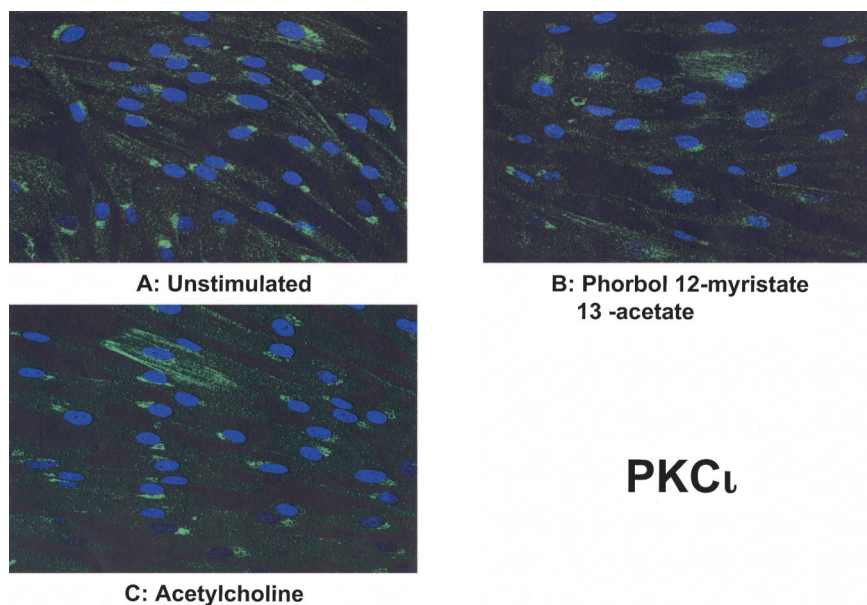
Fig. 6. Immunolocalization of protein kinase C (PKC)-ι. In unstimulated cells, PKC-ι exhibited a cytoplasmic fluorescent pattern, which was especially abundant in the perinuclear zones (*A*). No redistribution of PKC-ι was observed in cells exposed to phorbol 12-myristate 13-acetate (*B*). In acetylcholine-treated cells, the cytoplasmic staining pattern was similar to that of unstimulated cells (*C*).

smooth muscle contraction.[19-21] It has been reported that PKC isoforms mediate anesthesia-mediated contraction in various preparations.[22,23] In the current study, we identified four different isoforms of PKC in PVSMCs with diverse subcellular distribution. Moreover, we demonstrated translocation of PKC-α from cytoplasm to the membrane in response to acetylcholine. We also demonstrated that PKC inhibition attenuated acetylcholine contraction in PVSM. These data suggest that PKC-α translocation may partially mediate acetylcholine contraction in PVSM.

Role of PKC in Acetylcholine Contraction in PVSM

Vascular smooth muscle contraction is initiated by an increase in intracellular Ca^{2+} concentration. This results from an influx of Ca^{2+} across the sarcolemma through plasma membrane channels (*e.g.*, voltage-operated Ca^{2+} channels), as well as Ca^{2+} release from the sarcoplasmic reticulum (*e.g.*, inositol-1,4,5-trisphosphate–mediated Ca^{2+} release). However, vascular smooth muscle contraction is not simply proportional to changes in intracellular Ca^{2+} concentration, because Ca^{2+} sensitivity of the contractile apparatus is another important mechanism for vascular smooth muscle contraction.[24] Agonist-induced Ca^{2+} sensitization seems to be a G protein–mediated effect[25] and involves downstream effectors such as myosin light chain phosphatase,[26] PKC,[4,10] rho-kinase,[27] and tyrosine kinases.[28,29] Muscarinic receptors transduce their signals by coupling with G proteins. Acetylcholine binds to muscarinic receptors coupled to G_q, and activates phospholipase C-mediated hydrolysis of phosphatidylinositol-bis-phosphate to inositol 1,4,5-

A: Unstimulated

B: Phorbol 12-myristate 13 -acetate

C: Acetylcholine

PKCξ
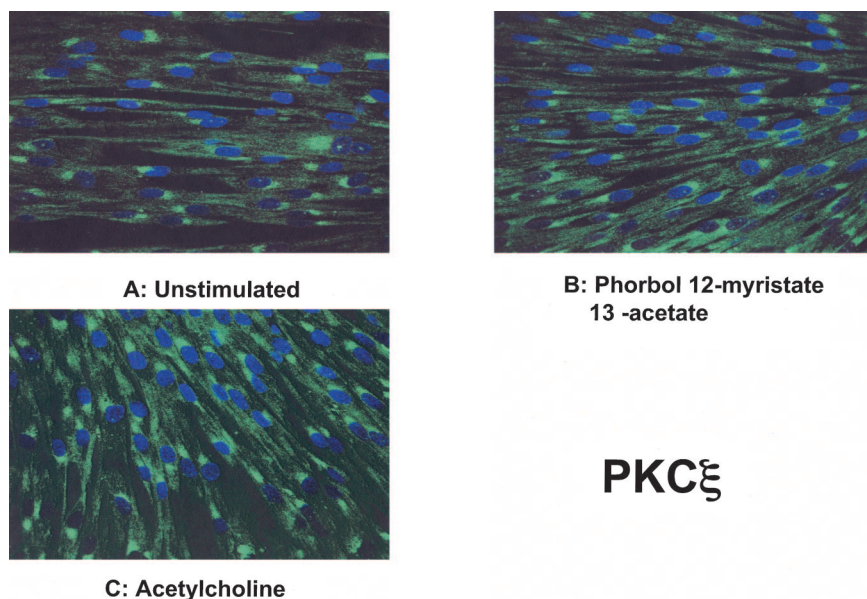
Fig. 7. Immunolocalization of protein kinase C (PKC)-ζ. In unstimulated cells, PKC-ζ exhibited a bright granular cytoplasmic fluorescent pattern, which was especially abundant in the perinuclear zones (*A*). No redistribution of PKC-ζ was observed in cells exposed to phorbol 12-myristate 13-acetate (*B*). In acetylcholine-treated cells, the cytoplasmic staining pattern was similar to that of unstimulated cells (*C*).

trisphosphate and diacylglycerol, which causes Ca^{2+} release and PKC activation, respectively. Recently, it has been reported that acetylcholine activates PKC and causes contraction in normal canine colonic circular muscle cells,[30] rat bronchial smooth muscle,[31] and cat esophageal smooth muscle.[32] We have previously demonstrated[4] that the rho kinase and tyrosine kinase pathways, as well as the PKC signaling pathway, mediate a component of acetylcholine contraction in pulmonary veins. The results presented in figure 1A confirm that PKC inhibition attenuates acetylcholine contraction. The observation (fig. 1B) that the conventional PKC isoform inhibitor, Gö6976, also attenuates acetylcholine contraction supports the concept that a conventional PKC isoform is involved in acetylcholine contraction, and that additional mechanisms are involved in this response.

Expression of PKC Isozymes in PVSMCs

Using isoform-specific antibodies in Western blotting experiments, we demonstrated the presence of PKC-α, -ι, -μ, and -ζ in PVSMCs. These results are consistent with numerous studies that report the expression of multiple PKC isoforms in smooth muscle. Multiple PKC isoforms have been reported in rat aorta,[33] ferret aorta,[34] and human saphenous vein and renal artery.[35] We have previously reported that PKC-α, -δ, -ε, -ι, -μ, and -ζ were expressed in canine pulmonary artery smooth muscle.[36] That study was the first to describe PKC isoform distribution in cells from the pulmonary circulation, and it was the first to report the presence of the ι/λ and μ isoforms of PKC in any type of vascular smooth muscle.[36] To our knowledge, the current study is the first to demonstrate the expression of PKC isoforms in PVSM. We observed that PKC-α, -μ, -ι, and -ζ are expressed in PVSMCs. In contrast to pulmonary artery smooth muscle,[36] we found that PKC-δ and -ε were not expressed in PVSMCs.

Acetylcholine Stimulation Causes PKC-α Translocation in PVSMCs

In the current study, we demonstrated by immunofluorescence stain that calcium-dependent PKC-α translocates from the cytosol to the membrane in response to acetylcholine in PVSMCs. PKC-α has been shown to translocate from the cytosol to the membrane when stimulated in a variety of cell types.[30,37] It has been proposed that translocation of PKC reflects PKC binding to intracellular receptors in the particulate fraction (RACKs) and that binding to RACKs may be required for PKC-mediated function.[38,39] PKC binding to RACKs is specific, dose-dependent, and saturable and may confer specificity of isoform action by differential localization of isoform-specific RACKs.[38,39] Acetylcholine-induced contraction was attenuated by conventional PKC isoform inhibition with Gö7936. Taken together, our results suggest that translocating PKC-α may be the isoform that

mediates a component of acetylcholine contraction in PVSM. One possible cellular mechanism could involve PKC-α–dependent activation of Na^+–H^+ exchange, which could cause intracellular alkalosis and a consequent increase in myofilament Ca^{2+} sensitivity.[40] In contrast, acetylcholine has no effect on the cellular distribution of the other PKC isoforms. The cellular function of these other isoforms in PVSM is not currently known.

Effect of PMA on Translocation of PKC Isoforms

Protein kinase C is a single polypeptide with an N-terminal regulatory region and a C-terminal catalytic region. Initial cloning of the conventional PKC group demonstrated that the polypeptide structure comprises four conserved (C1–C4) and five variable regions (V1–V5).[41,42] Regions C1 and C2 present at the N-terminal (approximately 20–70 kd) constitute the membrane-targeting regulatory domains that are required for interaction with diacylglycerol and phorbol esters, phosphatidylserine and Ca^{2+}. Incorporated within the C1 site are two cysteine-rich zinc fingers that comprise tandem C1A and C1B repeats that bind diacylglycerol and phorbol ester. By contrast, the C2 site is involved in Ca^{2+}-dependent membrane binding. Differences in the structure of the various PKC isoforms are associated mainly with the conserved region; the conventional PKC family contains each of the four conserved regions. The atypical PKCs lack the C2 region and have only one cysteine-rich loop in the C1 region. These structural differences result in the requirement of distinct cofactors for each of the PKC isoforms. Calcium and diacylglycerol are required to activate conventional PKCs. Atypical PKCs do not require these factors.[43] In the current study, PKC-α was observed to translocate from the cytosol to membrane in response to PMA, which is expected for isoforms in the conventional PKC subfamilies. In contrast, PKC-ζ, -ι, and -μ did not undergo PMA-induced translocation, which is expected for isoforms in the atypical PKC subfamilies.

Translocation of PKC Isoforms Requires Activation of Muscarinic Receptors

Classically, the activation of calcium-dependent PKC isoforms involves receptor-mediated activation of phospholipase C, resulting in generation of diacylglycerol and inositol 1,4,5-trisphosphate from membrane-associated phosphatidyl inositol[4,5]-bisphosphate. Subsequently, inositol 1,4,5-trisphosphate stimulates the release of intracellular calcium, which then binds to the C2 region of the PKC enzyme and promotes its translocation from the cytosol to the plasma membrane. In the current study, the muscarinic receptor agonist, acetylcholine, caused translocation of PKC-α from cytoplasm to membrane. The muscarinic receptor antagonist, atropine, had no effect on the cellular distribution of PKC-α but blocked the acetylcholine-induced translocation. These results

indicate that translocation of PKC-α by acetylcholine requires activation of muscarinic receptors.

In conclusion, acetylcholine contraction is attenuated by PKC inhibition in PVSM. Acetylcholine induces translocation of PKC-α from cytoplasm to membrane in PVSM. These results suggest that PKC-dependent acetylcholine contraction in PVSM may involve activation and translocation of PKC-α. This may be a fundamentally important cellular mechanism of pulmonary edema formation caused by pulmonary venous contraction. In future studies, we will investigate the extent and the cellular mechanism of action by which anesthetic agents alter the pulmonary vascular response to acetylcholine.

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