Selected isozymes of PKC contribute to augmented growth of fetal and neonatal bovine PA adventitial fibroblasts

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Das, Mita, Kurt R. Stenmark, Laura J. Ruff, and Edward C. Dempsey. Selected isozymes of PKC contribute to augmented growth of fetal and neonatal bovine PA adventitial fibroblasts. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1276–L1284, 1997.—We sought to determine which isozymes of protein kinase C (PKC) contribute to the increased proliferation of immature bovine pulmonary artery (PA) adventitial fibroblasts. Seven were identified in lysates of neonatal PA fibroblasts by Western blot: three Ca2+-dependent (α, βI, and βII) and four Ca2+-independent (δ, ε, ζ, and μ). Four isozymes (γ, η, θ, and ρ) were not detected in fibroblasts isolated at any developmental stage. Of the seven detected isozymes, only PKC-α - and -βII protein levels were higher in fetal and neonatal cells compared with adult fibroblasts. Their role in the enhanced growth of immature fibroblasts was then evaluated. The isozyme nonselective PKC inhibitor Ro-31-8220 was first compared with GF-109203X, a structural analog of Ro-31-8220 with relative specificity for the Ca2+-dependent isozymes of PKC. GF-109203X selectively inhibited the growth of immature cells and was nearly as potent as Ro-31-8220. Go-6976, a more specific inhibitor of the Ca2+-dependent isozymes, mimicked the antiproliferative effect of GF-109203X. PKC downregulation with 1 μM phorbol 12-myristate 13-acetate had the same selective antiproliferative effect on immature fibroblasts as GF-109203X and Go-6976. The protein levels of PKC-α - and -βII, but not of PKC-βI, were completely degraded in response to phorbol 12-myristate 13-acetate pretreatment. These results suggest that PKC-α - and -βII are important in the augmented growth of immature bovine PA adventitial fibroblasts.

protein kinase C-α; protein kinase C-βI; protein kinase C-ζ; protein kinase C-μ; Go-6976; GF-109203X; Ro-31-8220; phorbol 12-myristate 13-acetate-induced downregulation; pulmonary artery

ADVENTITIAL THICKENING of the pulmonary arteries is an important component of the structural changes observed in various forms of chronic pulmonary hypertension (17, 24, 26, 30). This thickening is due, at least in part, to proliferation of resident fibroblasts. The changes in proliferation of adventitial fibroblasts in response to injury appear more impressive in the neonatal than in the adult pulmonary circulation (9, 24, 26, 27, 30). In addition, recent experiments have demonstrated that the developmental differences in growth potential of bovine pulmonary artery (PA) adventitial fibroblasts (fetal > neonatal > adult) are also retained by isolated cells in vitro (2). However, the mechanisms contributing to the increased growth potential of immature adventitial fibroblasts remain poorly understood.

Protein kinase C (PKC) plays a key role in the regulation of cell proliferation, differentiation, and maturation (3, 6, 20). This pathway has been shown to be developmentally regulated (3, 23) and to contribute to the enhanced growth of neonatal bovine PA smooth muscle cells (3, 6). Activation of PKC increases responsiveness to developmentally regulated mitogens (3, 7) and is a requisite step for vascular cells to respond directly to hypoxia (4). In addition, this pathway has recently been implicated in the enhanced growth of fetal and neonatal PA adventitial fibroblasts by experiments demonstrating that immature fibroblasts have increased PKC catalytic activity and higher susceptibility to the growth-inhibiting effects of PKC antagonists compared with adult fibroblasts (2). The PKC signaling pathway, however, is a complex one, with four Ca2+-dependent and seven Ca2+-independent isozymes having been identified (12, 20). Developmental changes in the expression of individual isozymes have been observed, and increased expression of selected PKC isozymes has been linked to augmented growth capacity (23, 28, 32). However, the isozymes of PKC expressed by PA adventitial fibroblasts and their respective importance in the regulation of developmental differences in growth are not known.

The goals of this study were therefore to determine first the pattern of PKC isozyme expression that exists in PA adventitial fibroblasts and then to determine which specific isozymes contribute selectively to the increased growth potential of immature bovine PA adventitial fibroblasts (2). Our approach was to identify the PKC isozymes expressed by neonatal PA adventitial fibroblasts using antibody screening and then to quantitatively evaluate specific isozyme expression at different developmental stages. Finally, we used complementary antagonist strategies to determine whether two Ca2+-dependent isozymes, which were found to have increased expression during the fetal and neonatal period, contributed to the augmented growth of PA adventitial fibroblasts isolated at the same developmental stages.

Our data demonstrate that neonatal bovine PA adventitial fibroblasts express 7 of the 11 described isozymes of PKC as follows: three Ca2+-dependent (α, βI, and βII) and four Ca2+-independent (δ, ε, ζ, and μ). The expression pattern of two isozymes, the Ca2+-dependent PKC-α and -βII, paralleled the developmental differences in growth, susceptibility to PKC inhibitors, and PKC catalytic activity previously observed for PA adventitial fibroblasts (2). Antagonist strategies implicated these same Ca2+-dependent isozymes in the enhanced growth of fetal and neonatal PA fibroblasts. These observations suggest that PKC-α and -βII contribute to the augmented proliferative response of immature bovine PA adventitial fibroblasts.
MATERIALS AND METHODS

Materials. Minimal essential medium (MEM), trypsin-EDTA 10× suspension, penicillin, streptomycin, and amphotericin B were from Sigma Chemical (St. Louis, MO). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), and mercaptoethanol were also from Sigma. Anti-PKC-α, -γ, -ε, and -ζ antibodies and blocking peptides were purchased from Gibco BRL (Gaithersburg, MD). Anti-PKC-βI, -βII, -δ, -η, -θ, -λ, and -μ antibodies, blocking peptides, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) were from Santa Cruz Biotechnology (Santa Cruz, CA). Molecular mass markers and nitrocellulose membranes were obtained from Gibco BRL and Bio-Rad Laboratories (Richmond, CA), respectively. Enhanced chemiluminescence detection kits were from Amersham (Arlington Heights, IL). Reagents for protein determination were purchased from Bio-Rad Laboratories. Phorbol-12-myristate-13-acetate (PMA), Ro-31-8220, and GF-109203X were obtained from LC Services (Waltham, MA). Go-6976 was kindly provided by Parke-Davis Pharmaceutical Research (Ann Arbor, MI). All were dissolved in dimethyl sulfoxide (DMSO) and diluted to working concentrations in phosphate-buffered saline (PBS).

Isolation and growth of fetal, neonatal, and adult bovine PA adventitial fibroblasts. PA adventitial fibroblasts were isolated from 120- to 180-day-old bovine fetuses, 8- to 14-day-old neonatal calves, and adult cows, grown, and characterized as previously described (2). All cells were maintained in MEM, pH 7.4, supplemented with 10% serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and incubated in a humidified atmosphere with 5% CO2 at 37°C. Medium was changed biweekly. Cells were harvested weekly (i.e., passed) with trypsin (0.2 g/l)-EDTA (0.5 g/l). Early-passage (passages 1–6) cells were used. During the period of study, the cells retained stable growth properties and light-microscopic appearance.

Preparation of whole cellular lysates. Fetal, neonatal, and adult PA adventitial fibroblasts (0.5–2.0 × 10^6/flask) were plated in 15 ml of 10% serum-containing medium in T75 flasks. Cells were grown for 4–5 days under serum-stimulated conditions. Fibroblasts were washed with ice-cold PBS three times and were lysed in 1 ml of homogenization buffer [20 mM tris(hydroxymethyl)aminomethane, pH 7.5, containing 0.25 M sucrose, 3 mM EDTA, 3 mM ethylene glycol-bis(β-aminethoxyethyl) ether-N,N,N',N'-tetraacetic acid, 50 mM mercaptoethanol, 50 µg/ml leupeptin, 50 µg/ml aprotinin, 1 mM PMSF, and 0.1% Triton X-100] by freezing and thawing one time. On ice, each monolayer of PA adventitial fibroblasts was scraped into the homogenization buffer and triturated 10 times. Homogenates were centrifuged at 2,200 revolutions/min for 10 min at 4°C (Beckman centrifuge GS-6R; Fullerton, CA), and supernatant aliquots were immediately frozen in liquid nitrogen and stored at −80°C. Protein concentrations for each lysate were determined by micro-Bradford assay as previously described (3).

Western blot analysis of PKC isozymes. Twenty micrograms of each lysate were applied per slot for electrophoresis in each 10% reduced sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane. Prestained molecular mass protein markers were also loaded onto each gel. After a 2-h incubation at room temperature in 5% dry milk-PBS-0.05% Tween 20 to block nonspecific binding, the nitrocellulose was probed with a 1:300–500 dilution of primary PKC antibody-specific antisera in 5% milk-PBS-0.05% Tween 20 overnight at 4°C. Eleven different PKC isozyme-specific antisera were used. The nitrocellulose was then washed for 7 min three times with PBS-0.05% Tween 20. To detect bound primary antibody, blots were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG at a dilution of 1:5,000 in 5% milk-PBS-0.05% Tween 20. The nitrocellulose was washed again with PBS-0.05% Tween 20 for 5 min three times and then with PBS for 5 min one time. Blots were developed onto Dupont reflection autoradiography film (Dupont, Wilmington, DE) using an enhanced chemiluminescence detection kit. Determination of molecular mass for each isozyme detected was made by comparison with known molecular mass markers. Developmental and downregulation-induced changes in isozyme expression were determined by comparing matched cell lysates run side by side. Band intensity was quantified by scanning with a Lacie-limited Silverscanner II and by analysis with National Institutes of Health (NIH) Image Software (NIH, Bethesda, MD). Data were expressed as a percent of the fetal value.

To confirm the specificity of binding between primary antibody and immunoreactive protein, Western blots were performed in the presence and absence of isozyme-specific immunizing peptide. Isozyme-specific antisera were preincubated with the respective peptide antigen (1:10) used for immunization for 2 h at room temperature or overnight at 4°C and were then diluted to working concentration as described above. When an isozyme could not be detected in PA adventitial fibroblasts isolated at any developmental stage, positive control lysates were used to establish reactivity of the antibody [e.g., rabbit brain (γ), rat lung (η), skeletal muscle (θ), and bovine PA smooth muscle cells (μ)]. Specificity of the antibody was then confirmed as described above.

Differences in susceptibility to the antiproliferative effects of a nonselective PKC antagonist vs. PKC antagonists selective for the Ca^{2+}-dependent isozymes. Cells were sparsely seeded (10 × 10^5/well of a 24-well plate) in MEM-10% serum and allowed to attach overnight. On day 1, cell numbers were measured by hemocytometer to ensure that equal numbers were being compared. Then Ro-31-8220 (3 µM), an isozyme nonselective PKC inhibitor (13), GF-109203X (3 µM), an inhibitor with relative specificity for the Ca^{2+}-dependent isozymes (31), or the appropriate vehicle control was added. In preliminary studies, the dose-dependent inhibitory effects of the two related bisindolylmaleide compounds Ro-31-8220 and GF-109203X were first compared on the fastest growing cell population (fetal PA adventitial fibroblasts). Threshold and maximal antiproliferative effects for both compounds were found with 1 and 5 µM concentrations, respectively. Therefore, an intermediate (3 µM) concentration was used for the comparison between different cell populations in the current studies. This is the same concentration range for these compounds that others have applied to nonvascular cells (10) and that we have recently used for studies on PA smooth muscle cells (33). A close correlation between the extent to which PKC-mediated phosphorylation events are inhibited and the extent to which the cell response of interest is blocked in the presence of inhibitor has previously been observed (10). To confirm that the selective antiproliferative effects of GF-109203X were due to inhibition of the Ca^{2+}-dependent isozymes of PKC, 3 µM Go-6976 [a structural analog with greater specificity for these isozymes (15)] was also tested on the different cell populations. Mukherjee et al. (18) have recently used Go-6976 at the same concentration to implicate one of the Ca^{2+}-dependent isozymes of PKC in the regulation of phosphatidyethanolamine hydrolysis in MCF7 breast carcinoma cells. Inhibitors were
readded on day 3. Final cell numbers were counted on day 5. Results are expressed as cell number times 10^3 per well.

Differences in susceptibility to the antiproliferative effects of phorbol ester-induced PKC downregulation and detection of isozyme-specific differences in susceptibility to PKC downregulation. To detect developmental differences in susceptibility to the antiproliferative effects of phorbol ester-induced downregulation of PKC, cells were sparsely seeded (10^5–10^6 cells per well) in MEM-10% serum and allowed to attach overnight. Downregulation of PKC was achieved as previously described (2). On day 1, PA fibroblasts were pretreated with either 1 µM PMA or vehicle (DMSO) alone for 24 h. Change in cell number was then measured between days 2 and 5. Because the interval between PMA pretreatment and final growth measurement was long (72 h) and normal levels of PKC are gradually restored several hours after removal of PMA, the phorbol ester was routinely left in for the duration of the study (2). A repeat dose of PMA was applied on day 3 to be sure adequate levels were maintained. The results are expressed as cell number times 10^3 per well.

To determine the effect of PKC downregulation on isozymes of PKC, 1–2 × 10^6 neonatal PA fibroblasts were seeded in 10% serum-containing medium per T-75 flask and were grown for 4–5 days. Cells were then treated with either 1 µM PMA or vehicle (DMSO) alone for 24 h, and whole cell lysates were prepared as described above. PKC catalytic activity was measured in lysates of control and downregulated cells as previously described using myelin basic peptide (4–14) as substrate, with minor modifications (2). Change in isozyme expression was determined by Western blotting.

Data analysis. All data are presented as arithmetic means ± SE. For detection of PKC isozymes in neonatal PA fibroblasts, representative blots are shown. Results were reproduced at least three times in three different cell populations for molecular mass determinations. For studies of developmental change in expression of PKC isozymes with age, n equals the number of experiments done on at least three different populations of cells, each isolated from a different animal. For growth studies, n equals the number of replicate wells per test condition in representative experiments. Each observation was reproduced in cells isolated from at least three different animals. One-way analysis of variance followed by Student-Newman-Keuls multiple comparison test was used for these individual comparisons within and between groups of data points. Data were considered significantly different at P < 0.05.

RESULTS

Neonatal bovine PA adventitial fibroblasts express Ca^{2+}-dependent and -independent isozymes of PKC. To determine which isozymes of PKC are expressed in neonatal bovine PA adventitial fibroblasts, cell lysates were analyzed for immunodetectable proteins using Western blotting techniques and polyclonal antibodies against 11 isozymes of PKC. Specificity of the antibodies for each isozyme was demonstrated using corresponding blocking peptides. Neonatal cells were found to express seven isozymes of PKC as follows: three Ca^{2+}-dependent (α, β, and βII) and four Ca^{2+}-independent (δ, ε, ζ, and μ; Fig. 1). PKC-βI, -ζ, and -μ were resolved as protein doublets. Their apparent molecular masses were: α, 82 ± 3; βI, 89 ± 4 and 80 ± 3; βII, 89 ± 4; δ, 78 ± 1; ε, 103 ± 7; ζ, 85 ± 3 and 69 ± 2; and μ, 113 ± 8 and 106 ± 7 kDa (n = 3). The apparent molecular mass for each isozyme is in agreement with previously reported values in cells selectively overexpressing each isozyme (7, 12, 20). The antibody for PKC-δ also detected a specific band of higher than expected molecular mass (105 kDa) that was extinguished by pre-treatment with the appropriate blocking peptide. Because of the magnitude of the molecular mass difference (105 vs. 78 kDa), this signal is unlikely to be a phosphorylated derivative of PKC-δ. It is more likely to be an unrelated peptide sharing the same antigenic determinant.

The following four isozymes of PKC were not detected in neonatal PA fibroblasts: one Ca^{2+} dependent (γ) and three Ca^{2+} independent (η, θ, and υ; Fig. 2). In each instance, activity and specificity of the anti-PKC antibody was confirmed with an appropriate positive control lysate, application of blocking peptide, and apparent molecular mass determination. Lysates prepared from fetal and adult cells were also tested for these four isozymes. No immunodetectable γ, η, θ, or υ isozyme was found at any developmental stage.
Expression of the Ca\(^{2+}\)-dependent \(\alpha\) and \(\beta_{II}\) isozymes of PKC is higher in immature PA adventitial fibroblasts than in adult cells. To determine if there were isozyme-specific changes in expression with advancing developmental stage that paralleled the differences in growth and catalytic activity previously observed (2), three sets of matched fetal, neonatal, and adult cell lysates were directly compared. Each lysate was from a different animal. There was higher expression of the Ca\(^{2+}\)-dependent \(\alpha\) and \(\beta_{II}\), but not \(\beta_{I}\), isozymes of PKC in immature PA fibroblasts compared with adult cells (Fig. 3, A and B). PKC-\(\beta_{II}\) peptide was not detectable in adult fibroblasts. There was no effect of age on the expression of Ca\(^{2+}\)-independent PKC-\(\delta\), -\(\epsilon\), and -\(\zeta\) in bovine PA adventitial fibroblasts (Fig. 4, A and B). PKC-\(\mu\) had increased expression in adult cells (Fig. 4, A and B).

The Ca\(^{2+}\)-dependent isozymes of PKC contribute to the augmented growth of immature bovine PA adventitial fibroblasts. To test whether the Ca\(^{2+}\)-dependent isozymes of PKC contribute to the augmented growth of

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**Fig. 2.** Neonatal bovine PA adventitial fibroblasts do not express the Ca\(^{2+}\)-dependent \(\gamma\) or Ca\(^{2+}\)-independent \(\eta\), \(\theta\), and \(\iota\) isozymes of PKC. Lysates from neonatal fibroblasts (FIB) and control tissues (rabbit brain, rat lung, rat skeletal muscle, and PA smooth muscle cells (SMC)) were subjected to SDS-PAGE and immunoblot analysis with PKC isozyme-specific (\(\gamma\), \(\eta\), \(\theta\), and \(\iota\)) antibodies. Two bands are detected in the rabbit brain control with anti-PKC-\(\gamma\) antibody, but only one band at the appropriate MW was extinguished by the blocking peptide. Antibodies for PKC-\(\eta\), \(\theta\), and \(\iota\) detected single bands in control lysates that were at the appropriate MW and were extinguished by blocking peptide. Nonspecific binding to high MW proteins in fibroblast lysates was noted with anti-PKC-\(\gamma\) and \(\theta\) antibodies. Positions of the molecular mass standards (kDa) are indicated on left. Data are representative of results from 3 separate experiments, each performed on neonatal fibroblasts from different calves. These isozymes were also not detected in lysates of fetal and adult cells.

**Fig. 3.** Expression of the Ca\(^{2+}\)-dependent \(\alpha\) and \(\beta_{II}\) isozymes of PKC is higher in immature PA adventitial fibroblasts than in adult cells. A: representative immunoblots for each Ca\(^{2+}\)-dependent isozyme. Whole cell lysates (20 µg) of fetal (F), neonatal (N), and adult (A) PA adventitial fibroblasts were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-PKC-\(\alpha\), -\(\beta_{I}\), and -\(\beta_{II}\) antibodies. B: quantitative analysis of expression pattern for each Ca\(^{2+}\)-dependent isozyme. Results are pooled from 3 separate experiments (n = 3). For each experiment, cells from different animals at each developmental stage were used. Values are expressed as percent of the fetal value. *P < 0.05 compared with fetal cells and **P < 0.05 compared with fetal and neonatal cells.
immature PA adventitial fibroblasts, the antagonistic effects of the specific, but isozyme nonselective, PKC inhibitor Ro-31-8220 (3 µM) were compared with GF-109203X (3 µM), a structural analog with relative specificity for the Ca²⁺-dependent isozymes of PKC (13, 31; Fig. 5, A and B). GF-109203X selectively inhibited serum-stimulated growth of fetal and neonatal, but not adult, cells and was nearly as potent as Ro-31-8220. To be certain that the selective antiproliferative effects of GF-109203X were due to inhibition of the Ca²⁺-dependent isozymes of PKC, Go-6976, a more specific inhibitor of these isozymes, was also used (15, 18). Go-6976 (3 µM) had the same selective inhibitory effect on growth of the immature cells as GF-109203X (Fig. 6A).

The Ca²⁺-dependent α and βII isozymes of PKC contribute to the augmented growth of immature bovine PA adventitial fibroblasts. Phorbol-induced PKC downregulation was found to have the same selective inhibitory effect as GF-109203X and Go-6976 on the augmented growth of immature PA adventitial fibroblasts (Fig. 6B). Isozymes of PKC may differ in their susceptibility to downregulation (11, 22, 23). Those that are susceptible can be implicated in the cellular response (i.e., enhanced growth) blocked by this antagonist strategy. Therefore, we identified which Ca²⁺-dependent isozymes in neonatal PA adventitial fibroblasts were susceptible to degradation with prolonged exposure to PMA (Fig. 7A). Phorbol pretreatment resulted in ~95% degradation of PKC-α and -βII, the same Ca²⁺-dependent isozymes that had increased expression in the immature PA fibroblasts. We also confirmed that the prolonged exposure to PMA caused a reduction in whole cellular PKC catalytic activity by 67 ± 3%. The same pattern of susceptibility to degradation was also observed for fetal and adult cells. In contrast, the principal band for PKC-βI was not susceptible to downregulation. Again, the same resistance to degradation was observed with fetal and adult cells.

Susceptibility of the Ca²⁺-independent isozyme PKC-µ to PMA-induced degradation was investigated next because of changes observed in expression of this isozyme with advancing developmental stage (Fig. 7B).

![Fig. 4. Expression of Ca²⁺-independent μ, but not δ, ε, or ζ, isozymes of PKC is regulated by development in PA adventitial fibroblasts. A: representative immunoblots for each Ca²⁺-independent isozyme. Immunoblots from fetal (F), neonatal (N), and adult (A) PA adventitial fibroblasts for PKC-δ, ε, ζ, and µ are shown. B: quantitative analysis of expression pattern for each Ca²⁺-independent isozyme. Results are pooled from 3 separate experiments (n = 3). For each experiment, cells from different animals at each developmental stage were used. Values are expressed as percent of the fetal value. *P < 0.05 compared with fetal and neonatal cells.](http://ajplung.physiology.org/)

![Fig. 5. Ca²⁺-dependent isozymes of PKC contribute to the augmented growth of immature PA adventitial fibroblasts; n = 4 replicate wells. A: antiproliferative effects of Ro-31-8220, a specific but isozyme nonselective inhibitor of PKC. B: antiproliferative effects of GF-109203X, a structural analog with relative specificity for Ca²⁺-dependent isozymes of PKC. GF-109203X (3 µM) selectively inhibited the enhanced growth of immature fibroblasts and was nearly as potent as Ro-31-8220 (3 µM). Cell counts were performed on day 5 after the initial application (and 2 days after reapplication) of either Ro-31-8220 or GF-109203X. Same vehicle [dimethyl sulfoxide (DMSO)] was used for both inhibitors and all test conditions. *P < 0.05 compared with control cells. Similar results were reproduced in 2 other matched sets of cell populations.](http://ajplung.physiology.org/)
Similar results were reproduced with 2 other matched sets of cell populations. Days 2 and 5 serum-stimulated growth of fetal and neonatal, but not of adult, fibroblasts. Cell counts were performed on day 5 after the initial application (and 2 days after reaplication) of the inhibitor. DMSO was used as the vehicle. *P < 0.05 compared with control cells. Similar results were reproduced with 2 other matched sets of cell populations.

Part of the growth response of each fetal, neonatal, and adult population was not dependent on developmental stage and was inhibited by Ro-31-8220 but not by GF-109203X, Go-6976, or PMA-induced downregulation. Therefore, we also determined which of the Ro-31-8220-sensitive, Ca²⁺-independent isozymes (δ, ε, and ζ) were resistant to degradation induced by prolonged pretreatment with PMA (Fig. 7B). Only PKC-ζ was not degraded after pretreatment with PMA, suggesting that this isozyme might contribute to an equal extent to the serum-stimulated growth of both immature and mature cells (but not to the enhanced growth of the immature fibroblasts).

DISCUSSION
In this study, we have demonstrated that neonatal bovine PA adventitial fibroblasts express the following seven isozymes of PKC: three Ca²⁺-dependent (α, βI, and βII) and four Ca²⁺-independent (δ, ε, ζ, and μ). Four isozymes were not detected at any of the three developmental stages investigated. Of the isozymes detected, only two (α and βI; both Ca²⁺-dependent) had higher levels in immature fibroblasts than in adult cells. This pattern of expression paralleled the developmental differences in growth and PKC catalytic activity previously observed for adventitial fibroblasts (2). These same isozymes of PKC were further implicated in the enhanced growth of immature PA fibroblasts by studies showing that GF-109203X, a PKC inhibitor with relative specificity for the Ca²⁺-dependent isozymes of PKC (31), inhibited the serum-stimulated growth of fetal and neonatal, but not adult, cells. It was nearly as potent as Ro-31-8220, a structural analog lacking isozyme specificity (13) that inhibited growth of all three cell types. The discrete antiproliferative effects of GF-109203X on immature PA adventitial fibroblasts were then reproduced with the same concentration of Go-6976, a related analog and more specific inhibitor of the Ca²⁺-dependent isozymes of PKC (15, 18). Phorbol ester-induced PKC downregulation was found to have the same selective antiproliferative effect on immature PA adventitial fibroblasts as GF-109203X and Go-6976.
The prolonged exposure to PMA induced near-complete degradation of immunodetectable PKC-α and -βII, but not of PKC-βI, in PA adventitial fibroblasts. Thus, based on initial isozyme detection, selective changes in expression with advancing developmental stage, and antagonist strategies that target catalytic activity and expression level, we have implicated both PKC-α and -βII in the augmented growth of immature bovine PA adventitial fibroblasts.

Using 11 different isozyme-specific antibodies, we have found that bovine PA adventitial fibroblasts express more isozymes of PKC than previously described in other fibroblast cell lines. With a smaller number of antibodies, rat fibroblasts were found to express only two (α and δ, but not βI, βII, or γ) or at most four (α, ε, δ, and ζ, but not β and γ) isozymes of PKC (1, 16). Human skin fibroblasts were also shown to express the same four PKC isozymes (α, ε, δ, and ζ, but not βI, βII, or γ; see Ref. 25). PKC-β mRNA was not detected in R6 rat embryonic fibroblasts (1). In contrast, in bovine PA fibroblasts, we have detected both PKC-βI and -βII, alternative splicing products of the same transcript. Two Ca²⁺-independent isozymes, PKC-η and -ζ, that have been detected in adjacent PA smooth muscle cells (5) were not detected in adventitial fibroblasts at any developmental stage. Finally, we report the presence of PKC-μ for the first time in vascular cells.

We have demonstrated that selective changes in expression of the Ca²⁺-dependent isozymes PKC-α and -βII occur in PA adventitial fibroblasts during development. The pattern parallels previously observed growth properties, susceptibility to PKC inhibitors, and catalytic activity of PKC for fetal, neonatal, and adult PA adventitial fibroblasts (2). This observation suggests that specific isozymes may have unique roles in the control of cell growth in developing vessels. These same isozymes have been implicated in specific responses in other cell systems (5, 19). Our work complements recent studies in the developing rat glomerulus and heart where the expression of PKC isozymes has also been shown to be regulated in an age-dependent fashion. Saxena et al. (29) studied the role of PKC-β in the developing rat glomerulus. Differential expression of PKC-βII was found to parallel the proliferative behavior of maturing mesangial cells. Like our studies, their work suggests that PKC-βII expression and activation may play a critical role in development. Puceat et al. (23) found a selective decrease in expression of PKC-ζ in adult rat cardiomyocytes compared with neonatal cells. Rybin and Steinberg (28) reported an age-dependent decline in immunodetectable PKC-α and -δ as well as in PKC-ζ in developing rat heart. Expression of PKC-δ and -ζ did not change in our bovine PA cells. Thus developmental regulation of PKC isozymes appears to be cell, organ, and perhaps species specific, and different isozymes are probably serving unique roles in different cell populations.

We have implicated the two Ca²⁺-dependent isozymes α and βII in the enhanced growth of PA fibroblasts with complementary antagonist strategies. First, we compared the antagonistic effects of Ro-31-8220 and GF-109203X. Ro-31-8220 is a specific but isozyme-nonselective inhibitor (13), whereas GF-109203X, a related analog of Ro-31-8220, has relative specificity for the Ca²⁺-dependent isozymes of PKC (31). By comparing the antiproliferative effects of Ro-31-8220 and GF-109203X on the growth of PA smooth muscle cells, the Ca²⁺-dependent isozymes of PKC have recently been implicated in the enhanced growth of immature smooth muscle cells (33). In the current study, serum-stimulated growth of fetal and neonatal PA fibroblasts was selectively inhibited by GF-109203X. The antiproliferative effects of Ro-31-8220 on the immature cells were only slightly greater than those observed with GF-109203X. In adult cells, Ro-31-8220, but not GF-109203X, inhibited serum-stimulated growth. In contrast, adult PA smooth muscle cells were resistant to both inhibitors (33). Therefore, the effect of Ro-31-8220 on the adult fibroblast is cell-type specific. The differential antiproliferative effect of GF-109203X on immature PA fibroblasts was also reproduced with a specific inhibitor of the Ca²⁺-dependent isozymes of PKC, Go-6976 (15, 18). These results suggest that the Ca²⁺-dependent isozymes of PKC play an important role in the enhanced growth of immature PA fibroblasts. The selective pattern of the growth inhibition also shows that the results are not due to nonspecific effects of the bisindolmaleimide derivatives. The fact that one of the compounds, Ro-31-8220, is a potent inhibitor of all three cell types suggests that permeability to these lipophilic compounds is similar in all three cell populations. This is also supported by the observation that the extent of isozyme downregulation after pretreatment with PMA, another lipophilic cell-permeable compound, is the same in immature and mature cells.

PKC downregulation induced by prolonged incubation with PMA also selectively inhibited the growth of immature fibroblasts. This treatment is known to inhibit PKC catalytic activity and greatly reduces immunoreactive protein by proteolytic degradation (11). PKC isozymes have different susceptibility to this form of proteolytic degradation (5, 11, 22, 23). We took advantage of this phenomenon to determine which of the Ca²⁺-dependent isozymes of PKC in PA fibroblasts was depleted by this inhibitory strategy. In PA fibroblasts, there was selective reduction in the immunoreactive peptide levels of PKC-α and -βII, but not of PKC-βI, in response to the high concentration of PMA. Interestingly, these same two isozymes of PKC are the ones that are increased in immature cells. In other cell systems, PKC-α has been consistently found to be susceptible to phorbol ester-induced downregulation, although the results with PKC-βI have been more variable (14, 23).

We have found that expression of the Ca²⁺-independent isozyme PKC-μ is lower in immature cells compared with adult fibroblasts. Because isozymes of PKC could also exert a negative influence on proliferation, we considered the possibility that PKC-μ could be important in growth regulation. Johannes et al. (12) demonstrated that PKC-μ activity was inhibited by sphingosine but not by staurosporine. In our previous study, we observed that dihydro sphingosine had the
same differential effect on the growth of fetal, neonatal, and adult fibroblasts (2) as GF-109203X and Go-6976, which are staurosporine derivatives. There has been a recent report that very high concentrations of Go-6976 can partially inhibit the kinase activity of PKC-μ in intact cells (half-maximal inhibitory concentration = 20 μM; see Ref. 8). However, no inhibition was observed with 3 μM, the concentration used in our studies. Therefore, the selective antiproliferative effect of Go-6976 on immature cells cannot be attributed to inhibition of PKC-μ. We also demonstrated that PKC-μ is resistant to PMA-induced downregulation in vascular fibroblasts. A shift in phosphorylation state was observed but not degradation. This finding is consistent with a recent report on HeLa and COS cells stably transfected with PKC-μ as well as a carcinoma cell line expressing endogeneous PKC-μ (12). Collectively, these results suggest that PKC-μ is not responsible for the enhanced growth of immature PA fibroblasts.

Although it was not the focus of this paper, we were also able to compare results with the different antagonist strategies to establish a role for PKC-ζ in an additional component of growth of all three cell types that was not developmentally regulated and was Ro-31-8220 sensitive but GF-109203X, Go-6976, and PMA downregulation resistant. These results are consistent with the fact that PKC-ζ lacks a phorbol ester binding domain and has been implicated in the mitogenic response of some nonvascular cells (34). Interestingly, this isozyme has been localized to the nucleus in other cell systems (34), and by immunostaining we have found the same for PA adventitial fibroblasts (personal communication).

In summary, using antibodies against the 11 described isozymes of PKC, multiple Ca2+-dependent and -independent isofoms have been detected in neonatal bovine PA adventitial fibroblasts. The levels of two Ca2+-dependent isozymes, PKC-α and βII, have been found to be selectively higher in fetal and neonatal than in adult PA adventitial fibroblasts. The change in expression pattern of these two isozymes paralleled the differences in growth, PKC catalytic activity, and susceptibility to PKC inhibitors previously observed in these vascular cells with advancing developmental stage. Using complementary antagonist strategies, these same Ca2+-dependent (α and βII) isozymes of PKC have been implicated in the enhanced growth capacity of immature PA fibroblasts in vitro. Therefore, PKC-α and βII may also be important in the augmented proliferative response of neonatal bovine PA adventitial fibroblasts observed in vivo in settings of vascular injury.

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