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ARTICLES

Role of Protein Kinase C in Arginine Vasopressin-Stimulated ERK and p70S6 Kinase Phosphorylation

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Abstract We previously showed in rat renal glomerular mesangial cells, that arginine vasopressin (AVP)-stimulated cell proliferation was mediated by epidermal growth factor receptor (EGF-R) transactivation, and activation (phosphorylation) of ERK1/2 and p70S6 kinase (Ghosh et al. [2001]: Am J Physiol Renal Physiol 280:F972–F979]. In this paper, we extend these observations and show that different protein kinase C (PKC) isoforms play different roles in mediating AVP-stimulated ERK1/2 and p70S6 kinase phosphorylation and cell proliferation. AVP treatment for 0–60 min stimulated the serine/threonine phosphorylation of PKC isoforms α, δ, ε, and ζ. The activation of PKC was dependent on EGF-R and phosphatidylinositol 3-kinase (PI3K) activation. In addition, inhibition of conventional and novel PKC isoforms by chronic (24 h) exposure to phorbol 12-myristate 13-acetate (PMA) inhibited AVP-induced activation of ERK and p70S6 kinase as well as EGF-R phosphorylation. Rottlerin, a specific inhibitor of PKCδ, inhibited both ERK and p70S6 kinase phosphorylation and cell proliferation. In contrast, a PKCε translocation inhibitor decreased ERK1/2 activation without affecting p70S6 kinase or cell proliferation, while a dominant negative PKCζ (K281W) cDNA delayed p70S6 kinase activation without affecting ERK1/2. On the other hand, Gö6976, an inhibitor of conventional PKC isoforms, did not affect p70S6 kinase, but stimulated ERK1/2 phosphorylation without affecting cell proliferation. Our results indicate that PKCδ plays an important role in AVP-stimulated ERK and p70S6 kinase activation and cell proliferation. J. Cell. Biochem. 91: 1109–1129, 2004.

Key words: proliferation; EGF-R; PI3K

Arginine vasopressin (AVP) is a neuropeptide hormone that can modulate vascular tone and glomerular filtration through its contractile effects on glomerular mesangial cells [Venkatachalam and Kreisberg, 1985; Kreisberg and Wilson, 1988]. Additionally, it can induce hypertrophy and cell growth in both mesangial and smooth muscle cells [Ganz et al., 1988; Schulze-Lohoff et al., 1993]. AVP exerts different effects on different cell types, depending on the class of receptor the cells express (i.e., V1 or V2 receptors). These receptors are G-protein coupled receptors (GPCRs), i.e., receptors that couple to heterotrimeric G proteins. AVP exerts its actions in mesangial cells through binding to specific V1 Gq/phospholipase C (PLC)-coupled vascular receptors [Jard et al., 1987]. V1 receptor activation results in elevation of intracellular Ca^{++} and activation of PLC.

Protein kinase C (PKC) is activated as a consequence of PLC activation [Moriya et al., 1996]. Studies from our laboratory showed that upon binding to its specific V1 receptor, AVP activates a phosphatidylinositol specific-PLC (PI-PLC) in rat mesangial cells [Troyer et al., 1985, 1992]. This results in elevated levels of inositol trisphosphate (IP3) and diacylglycerol (DAG), leading to mobilization of intracellular stores of Ca^{++} and stimulation of PKC [Venkatachalam and Kreisberg, 1985; Bonventre et al., 1986]. Ca^{++} mobilization and PKC activation induced increased cell signaling

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leading to cell-cycle progression in mesangial and other cells [Ganz et al., 1990; Sjoholm et al., 2000]. Two isoforms of PLC have been described, PLCβ, which is activated by GPCRs, and PLCγ, whose activation may be dependent upon protein tyrosine kinase (PTK), which includes phosphatidylinositol 3-kinase (PI3K) or receptor tyrosine kinase (RTK) activation [Carpenter and Ji, 1999]. Thus, PKCs may be activated by either GPCRs or by PTKs.

The PKC superfamily comprises serine/threonine protein kinases found in mammalian cells; these distinct isoforms of PKC have been implicated in a multitude of cellular processes [Takai et al., 1979; Kishimoto et al., 1980; Nishizuka, 1995; Newton, 1997]. PKCs have been subdivided into three subfamilies according to their lipid-activation profiles: conventional PKCs (α, βI, βII, and γ) are activated by DAG and Ca^{++} [Takai et al., 1979; Kishimoto et al., 1980; Nishizuka, 1995; Newton, 1997]; novel PKCs (δ, ε, η, μ, and θ) do not respond to Ca^{++} but require DAG while atypical PKCs (ζ and λ/ι) are not activated by either DAG or Ca^{++} [Corbit et al., 2000]. All PKCs require the phospholipid phosphatidylserine as a cofactor. Rat mesangial cells express the conventional PKCα, the novel PKCs (PKCε and PKCδ), and the atypical PKCζ [Huwiler et al., 1993; Kikkawa et al., 1994]. In addition, the expression of the conventional PKCs βI and γ in mesangial cells have also been reported [Saxena et al., 1993] but were not detected in our cells [Kreisberg et al., 1996].

Phosphorylation on critical residues is necessary for the activation of all three classes of PKCs [Newton, 1997, 2003]. Novel and conventional PKCs require phosphorylation at three different sites in order to be activated by DAG [Keranen et al., 1995; Parekh et al., 2000]. In the first phosphorylation step, a residue in the activation loop of the carboxy terminal is phosphorylated by phosphoinositide-dependent protein kinase-1 (PDK-1), a downstream effector of PI3K [Cenni et al., 2002]. This step is required to correctly realign residues in the active site. Different PKC isoforms are phosphorylated at different residues in this step. In the case of PKCα, this residue is Thr 497 [Bornancin and Parker, 1996], while for PKCε, it is Thr 566 [Le Good et al., 1998]. PDK-1 also phosphorylates Thr 505 of PKCδ, and Thr 410 of PKCζ [Chou et al., 1998; Gliki et al., 2002]. This first phosphorylation step triggers autophosphorylation at two other sites in the catalytic domain, the first in the "turn motif," and the last in the "hydrophobic motif" [Dutil et al., 1998]. These sites are Thr 710 and Ser 729, respectively in PKCε [Cenni et al., 2002], Thr 638 and Ser 657 in PKCα [Bornancin and Parker, 1996], and Ser 643 and Ser 662 for PKCδ [Le Good et al., 1998; Parekh et al., 2000]. Phosphorylation at these sites primes the kinase for activation by DAG. In the case of sufficient supply of DAG and/or Ca^{++}, and without interruption of its binding to the different PKC isoforms, phosphorylation at these sites is an indicator of the activation of the PKC isoform investigated. Due to a lack of both a DAG binding domain and a Ca^{++} binding domain in atypical PKCs, these kinases are regulated solely by phosphorylation by PDK-1 [Newton, 2003]. PDK-1 dependent phosphorylation at Thr 410 is followed by autophosphorylation at Thr 560, which renders the kinase completely active [Standaert et al., 2001].

In this paper, we show that PKC is phosphorylated in response to AVP stimulation in rat mesangial cells. In addition, we show the importance of PI3K and the epidermal growth factor receptor (EGF-R) for activation of PKC in AVP-stimulated mesangial cell proliferation. Our data also show that PKCs play an important role in AVP-stimulated p70S6 kinase and ERK1/2 activation, EGF-R phosphorylation and cell proliferation.

MATERIALS AND METHODS

Cell Culture and Pharmacological Treatments

Rat mesangial cells were isolated and established in culture as previously described [Kreisberg and Wilson, 1988; Kreisberg et al., 1994; Kreisberg et al., 1996]. The cells were cultured in RPMI 1640 containing 20% fetal bovine serum and 1% antimycotic–antibiotic solution (Mediatech-Cellgro, Herndon, VA). AG 1478, LY 294002, Rottlerin, PKCε translocation inhibitor, and Gö6976 were purchased from Calbiochem (La Jolla, CA). AVP was from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti phospho-ERK (Thr 202/Tyr 204), anti-phospho-PKCδ(T505), anti-phospho-PKCδ(S643), and anti-phospho-p70S6 kinase (T421/S424) antibodies were from Cell Signaling Technology (Beverly, MA). Anti-PKCα, anti-PKCε, anti-PKCδ, anti-PKCζ, anti-phospho-PKCα(S657), anti-phospho-EGF-R(Y1173), and anti-phos-

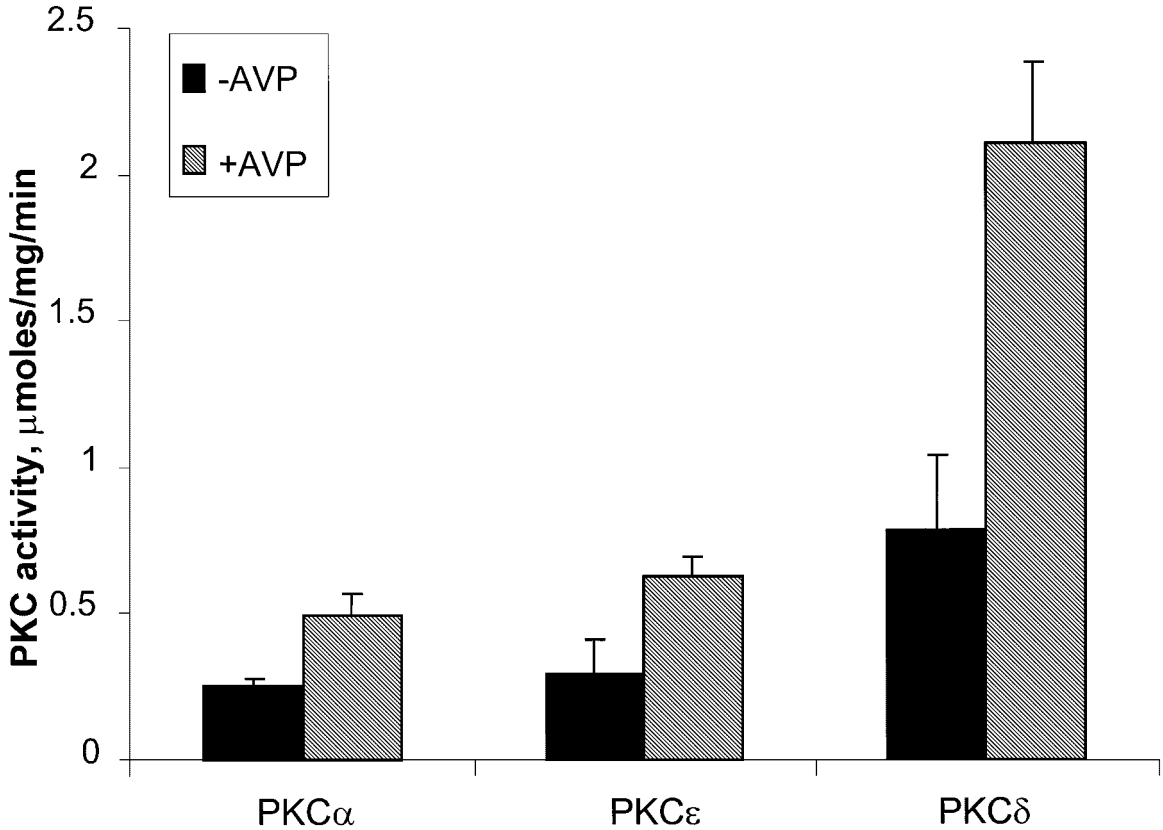


Fig. 1. Basal and arginine vasopressin (AVP)-stimulated protein kinase C (PKC) activity in rat glomerular mesangial cells. Cells were serum starved for 48 h prior to addition of PBS (control) or 1 μM AVP for 15 min for the detection of PKCα or PKCε or for 5 min for the detection of PKCδ activity. Cells were lysed in lysing buffer and immunoprecipitated with antibodies to PKCα, PKCε, or PKCδ. Immunoprecipitates were then assayed for PKC activity using a synthetic peptide substrate as described in Materials and Methods. Bar graphs represent the mean ± standard deviation (SD) from four individual experiments.

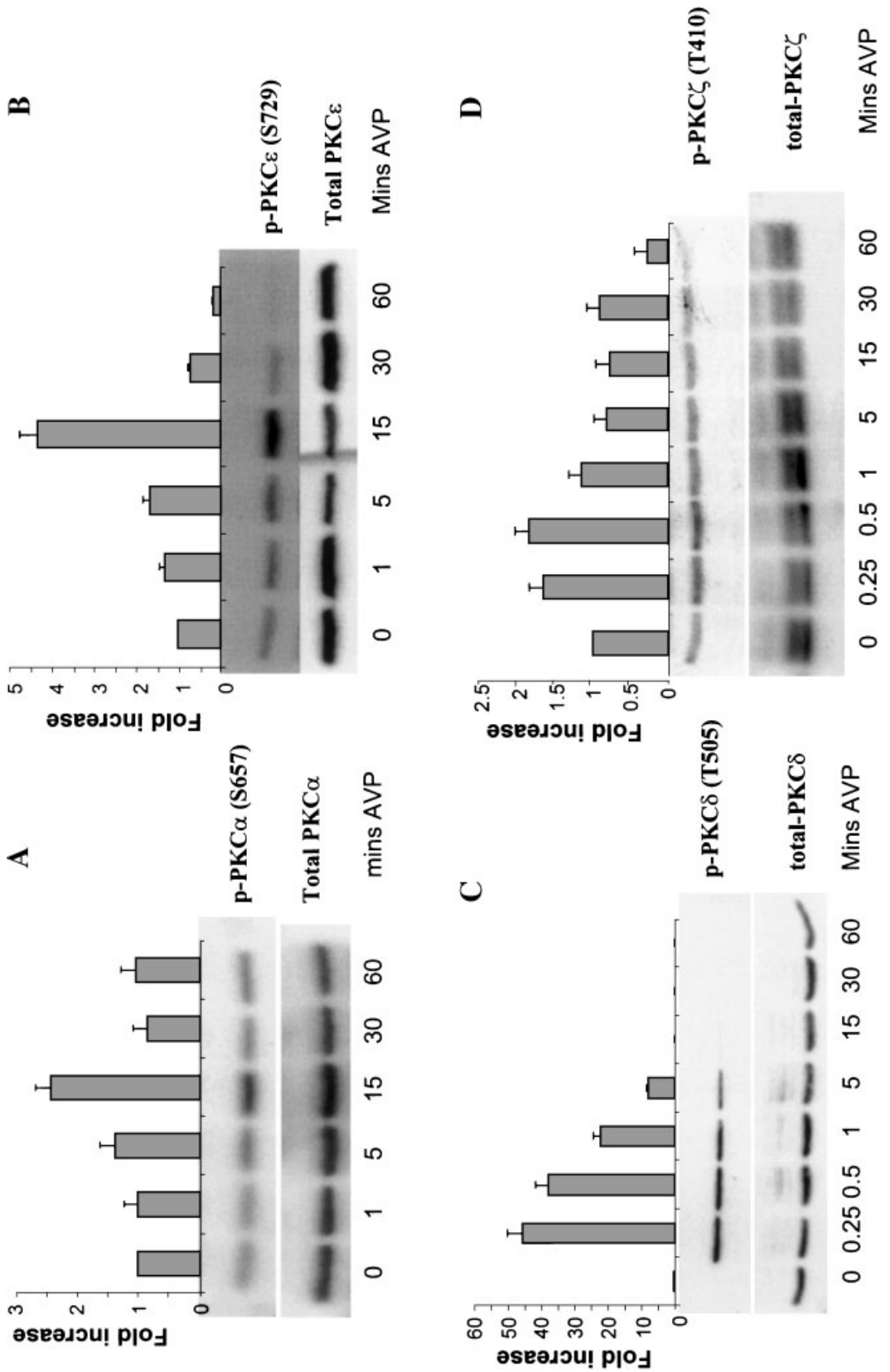
pho-PKCε(S729) were from Upstate Biotechnology (Lake Placid, NY). Two rabbit polyclonal anti-phospho-PKCζ antibodies were used: the PKCζ(T410) antibody was generously donated by Dr. Alex Tucker, Harvard Medical School (Boston, MA), while another, the anti-phospho-PKCζ/λ (Thr 410/403) was purchased from Cell Signaling Technology. All other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

PKCε Translocation Inhibition

PKCε translocation was inhibited using a water-soluble octapeptide trifluoracetate salt (H–Glu–Ala–Val–Ser–Leu–Lys–Pro–Thr–OH) [Johnson et al., 1996]. The octapeptide was administered to the cells using the transient permeabilization method [Johnson et al., 1996]. Briefly, the conditioned medium was removed and saved at 37°C and the cells washed twice with PBS. Next, the cells were permeabilized in the presence of the octapeptide for 10 min with permeabilization buffer (20 mmol/L HEPES, pH 7.4; 10 mmol/L EGTA; 140 mmol/L KCl; 50 mg/ml saponin; 5 mmol/L NaN_3; 5 mmol/L oxalic acid dipotassium salt). Following incubation, the permeabilization buffer was removed and the cells washed five times with PBS. Next, the PBS was aspirated and the cells incubated in conditioned medium for 30 min at 37°C prior to AVP treatment.

Immunoblotting

Mesangial cells were grown on 100-mm dishes at 1,000,000 cells/dish and serum starved for 48 h prior to the experiments. Whole cell extracts were prepared by washing the cells twice in PBS and lysing cells in 250 μl RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.1% NP-40, protease inhibitors: 0.1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml each of phenathroline, leupeptin, aprotinin, and pepstatin A and phosphatase inhibitors: 20 mM B-glycerol


A
Fold increase
p-PKCα (S657)
Total PKCα
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0 1 5 15 30 60
B
Fold increase
p-PKCε (S729)
Total PKCε
Mins AVP
0 1 5 15 30 60
C
Fold increase
p-PKCδ (T505)
total-PKCδ
Mins AVP
0 0.25 0.5 1 5 15 30 60
D
Fold increase
p-PKCζ (T410)
total-PKCζ
Mins AVP
0 0.25 0.5 1 5 15 30 60

Fig. 2.

phosphate, 1 mM Na-orthovanadate, and 10 mM NaF). Proteins were quantitated using a BCA assay (Pierce, Rockford, IL) and fractionated on SDS–polyacrylamide gels (PAGE). Electrophoresis was performed at 45 mA for approximately 45 min using mini-vertical electrophoresis cells (Mini-PROTEAN II Electrophoresis Cell, BioRad, Hercules, CA). The gels were electroblotted for 1.5 h at 200 mA using a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) onto 0.2 μm PVDF membrane (Osmonics, Westborough, MA). The blots were stained with primary antibodies at a dilution of 1:500. The staining was detected by enhanced chemiluminescence (Pierce) after incubation with a peroxidase labeled secondary antibody. Following detection of a phospho-specific antibody, the blot was stripped using stripping buffer (Chemicon International, Temecula, CA) and re-probed for the corresponding total antibody. The blots were scanned and the bands quantitated using NIH Image 1.62 software on a Macintosh G3 computer. The band intensity of the phospho-specific antibody was divided by the corresponding band intensity of the total antibody, and the results expressed as fold increases over control. Each experiment was repeated three times.

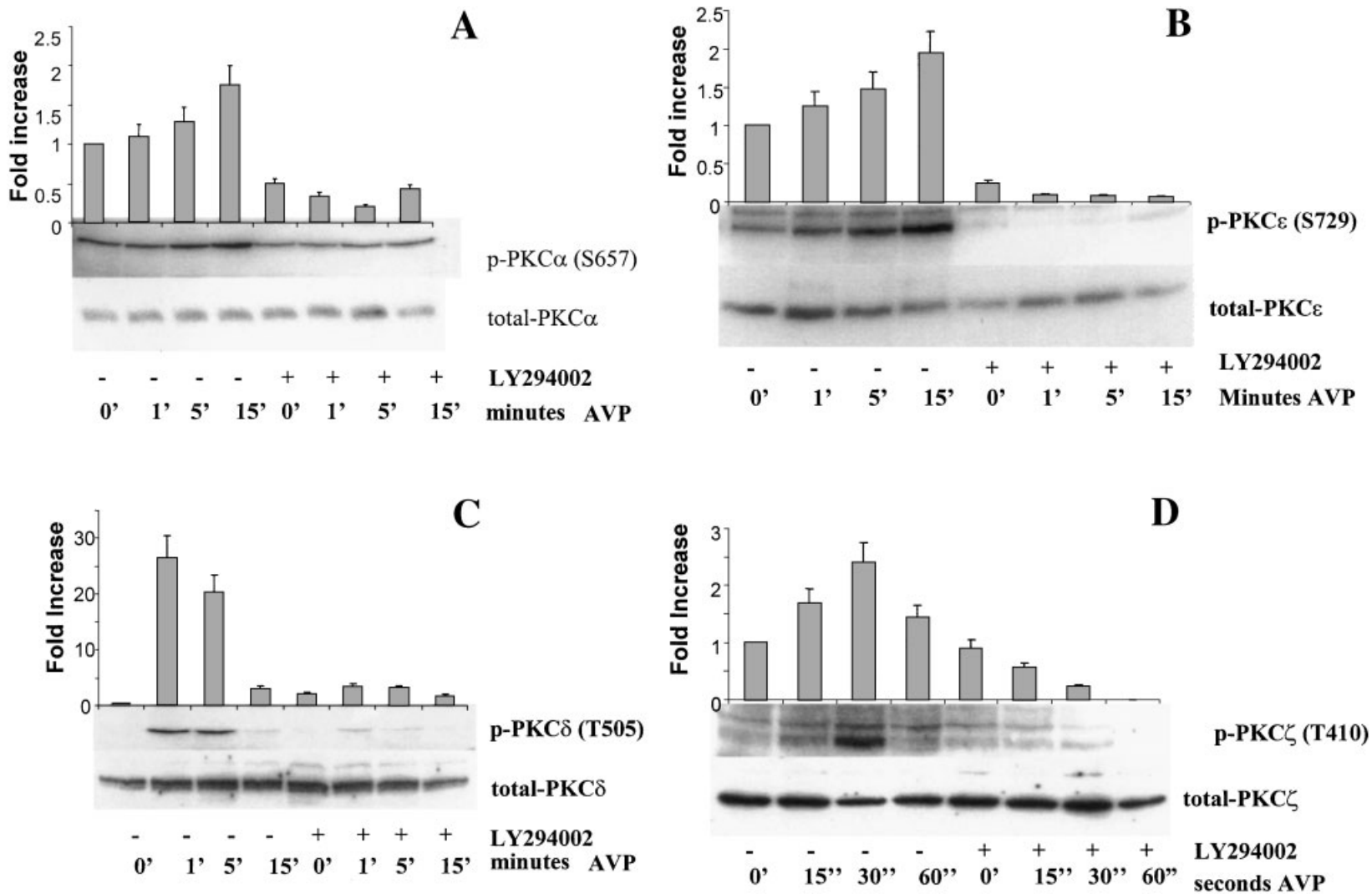
Transfections

Cells were transiently transfected using lipofectamine PLUS reagent (Invitrogen Corp., Carlsbad, CA) with 1 μg dominant negative PKCζ(K281W) cDNA (generously provided by Dr. Alex Toker, Harvard Medical School) and 100 ng of pEGFP-C1 (Clontech Laboratories, Inc., Palo Alto, CA), a vector containing the gene for green fluorescent protein (GFP) per 100 mm dish. Controls received the GFP plasmid alone. The DNA–lipofectamine mix was overlayed onto cells in RPMI for 3–4 h, after which the cells were washed and recovered in complete medium (RPMI 1640 with 20% FCS) for 14 h. Transfection efficiency was approximately 20–25% in cells transfected with GFP alone and 15–20% in cells transfected with GFP + plasmids as determined by flow cytometry. GFP-expressing cells were sorted by flow cytometry and transfectedcells (96–98% GFP expressing, as confirmed by repeat sorting) were collected in a tube containing culture medium and re-plated in tissue culture dishes for further experiments. All experiments were done at 48 h post-transfection.

Flow Cytometry

Cells to be processed for flow cytometry were trypsinized, then resuspended in 2 ml cell growth medium, and spun down at 1,000*g* for 5 min in a table top centrifuge. The medium was aspirated and the pellet washed once in PBS. Fluorescense analysis and cell sorting was performed using a FACStar Plus flow cytometer (Becton-Dickinson Immunocytometry Systems, Inc., San Jose, CA). Cells were illuminated with 250 mW of 488 nm laser light from an argon-ion laser (Coherent, Inc., Santa Clara, CA) and green fluorescence was read through a 530/30 nm bandpass filter. Data was collected, stored, and analyzed using a Macintosh G3 computer running CellQuest V3.1 software from Becton-Dickinson (Franklin Lakes, NJ). For cell-cycle analysis, trypsinized cells were fixed in 500 μl 70% ethanol, and incubated 30 min at −20°C. The fixed cells were repelleted, and washed twice in 1% BSA/PBS. Following

Fig. 2. Immunoblots stained with isotypic specific phospho-PKC antibodies after treatment for different time periods with AVP (1 μM). Mesangial cells were grown to confluence and serum starved for 48 h. AVP was added for 0–60 min. **A**: Activation of PKCα was demonstrated by an increase in phospho-PKCα (S657) using a phospho-specific antibody [p-PKCα(S657)]. PKCα showed increased phosphorylation with a peak at 15 min post-stimulation, after which the level of phosphorylation decreased. The blot was then stripped and restained with total PKCα antibody (**lower panel**) for loading control. **B**: Activation of PKCε was demonstrated by an increase in phosphorylation of PKCε (S729) using a phospho-specific antibody [p-PKCε(S729)]. PKCε also showed peak phosphorylation at 15 min post-stimulation. The blot was then stripped and restained with total PKCε antibody (**lower panel**). **C**: Activation of PKCδ was demonstrated by an increase in phosphorylation of PKCδ (T505) using a phospho-specific antibody [p-PKCδ(T505)]. Unlike the other PKC isoforms studied, PKCδ (T505) showed low basal phosphorylation levels, but a strong increase in phosphorylation at 15 s post-stimulation, after which the level of phosphorylation decreased to control levels by 15 min post-stimulation. **Lower panel** demonstrates the expression of total PKCδ as loading control. **D**: Activation of PKCζ was demonstrated by an increase in phosphorylation of PKCζ (T410) using a phospho-specific antibody [p-PKCζ(T410)]. PKCζ showed peak phosphorylation at 30 s post-stimulation. The blot was then stripped and restained with total PKCζ antibody (**lower panel**). For each of these figures, the bars represent the ratio of the intensity of the band obtained with the phospho-specific antibody (**upper panel**) to the intensity of the band obtained with the total antibody (lower panel). The numbers were normalized to controls, and the mean ± standard error (SE) obtained from three individual experiments.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0' 15'' 30'' 60'' 0' 15'' 30'' 60'' seconds AVP

Fig. 3.

this, the cells were suspended in 150 μl PBS, 50 μl of 1 mg/ml RNAse A (Sigma-Aldrich) and 100 μl of 100 μg/ml propidium iodide (PI, Sigma-Aldrich) and incubated overnight at 4°C. Red fluorescence (for PI) was read through a 630/22 nm band-pass filter. Data was collected on 20,000 cells as determined by forward and right angle light scatter and stored as frequency histograms; data used for cell-cycle analysis was then analyzed using MODFIT (Verity software, Topsham, ME). All experiments were repeated at least twice.

PKC Assay

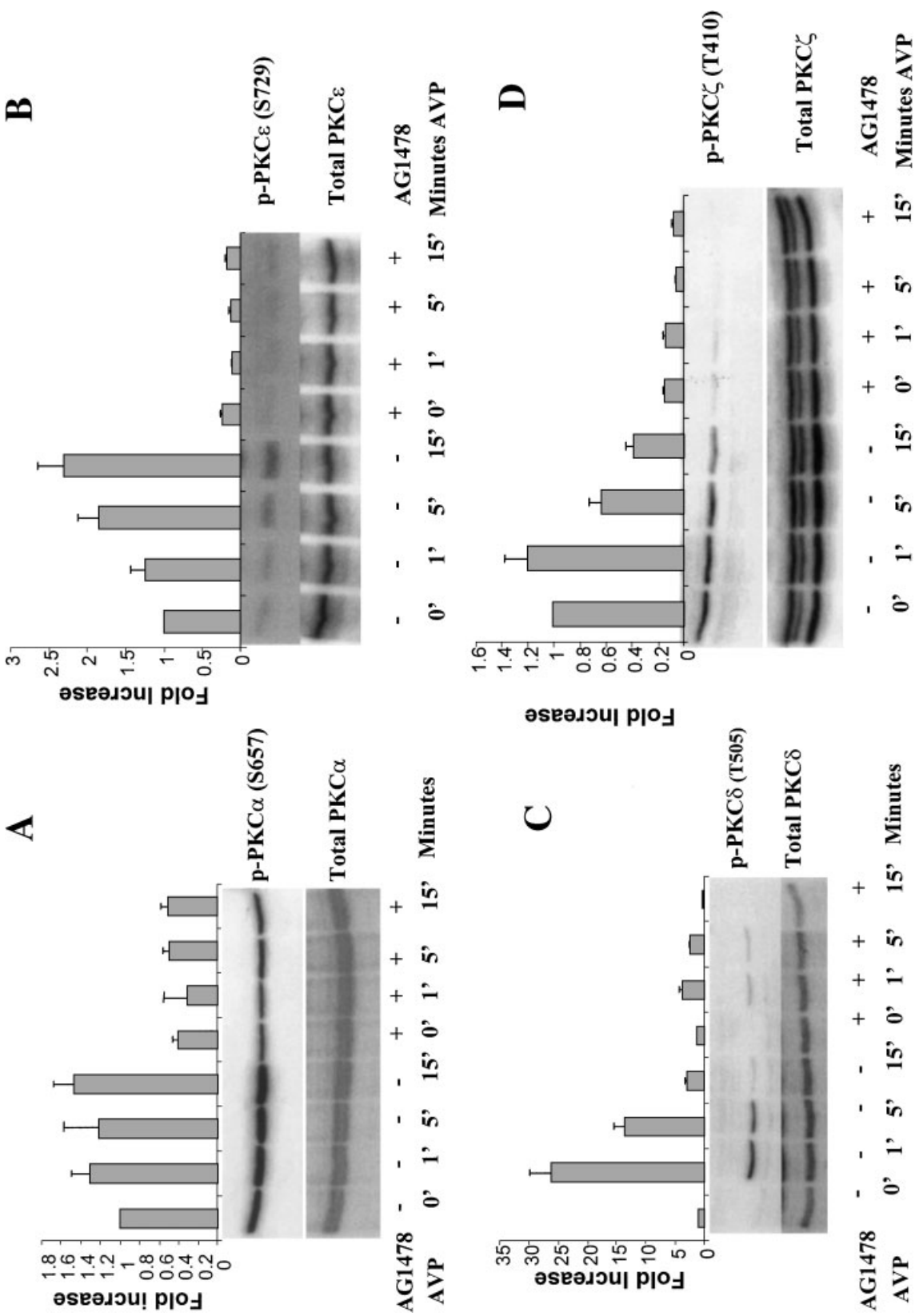
Determination of PKC activity is achieved with a commercially available kit according to manufacturer's instructions (Upstate Biotechnology). In brief, mesangial cells were plated on 100 mm tissue culture dishes and treated as desired. Five-hundred micrograms of protein obtained from whole cell lysates in cell lysis buffer (see Immunoblotting, above) was precleared with 25 μl of 50% protein A-sepharose beads in 400 μl of lysis buffer containing 1 μg/ml BSA for 1 h. The supernatants were incubated with the appropriate antibody (PKCα, PKCε, or PKCδ, 1–4 μg/sample) overnight. Next, 20 μl of protein A-sepharose was added for 1 h, the beads spun down and the immunocomplexes were washed three times with lysis buffer. For detection of PKC activity in the immunoprecipitates, 10 μl each of the following was added to the immunocomplexes: assay buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM CaCl2), 500 μM PKC substrate peptide [QKRPSQRSKYL] in the assay buffer, PKA/CaMK inhibitor cocktail (2 μM PKA inhibitor peptide, 20 μM Compound R24571 in assay buffer), PKC lipid activator (0.5 mg/ml phosphatidylserine, 0.05 mg/ml DAG in assay buffer). Next, 90 μl Mg^{2+}/ATP cocktail (75 mM $MgCl_2$, 500 μM ATP, 20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na_3VO_4, 1 mM dithiothreitol) was added to 100 μCi [γ-^{32}P]ATP to dilute it, and 10 μl of the diluted [γ-^{32}P]ATP mixture was added to each sample and incubated for 10 min at 30°C. Following incubation, 25 μl aliquot of the reactant was transferred onto phosphocellulose paper and the paper washed three times with 0.75% phosphoric acid and once with acetone, then loaded into scintillation vials and read. PKC activity was determined by calculating the specific binding of the phosphorylated substrate and expressed as micromoles phosphate incorporated into the PKC substrate peptide/min/mg enzyme. All experiments were performed in triplicate.

RESULTS

PKC Activity in Rat Renal Glomerular Mesangial Cells

We determined by Western blotting that rat mesangial cells in culture expressed PKC isoforms α, δ, ε, and ζ in confirmation with previous reports [Huwiler et al., 1993; Kikkawa et al., 1994]. We have previously shown that these cells do not express PKCβ or PKCγ [Kreisberg et al., 1996]. We immunoprecipitated mesangial cell lysates with polyclonal antibodies to PKCα, PKCε, and PKCδ and assayed the immunoprecipitates for PKC activity with a synthetic substrate peptide (QKRPSQRSKYL), as described in Materials and Methods. Our cells expressed high-basal levels of PKCδ activity (0.787 ± 0.248 μmoles phosphate incorporated into the PKC substrate peptide/min/mg enzyme) compared to basal levels of PKCα (0.252 ± 0.026 μmoles phosphate incorporated into the PKC substrate peptide/min/mg enzyme) and basal levels of PKCε (0.286 ± 0.131 μmoles phosphate incorporated into the PKC substrate peptide/min/mg enzyme) (Fig. 1). Vasopressin stimulates PKC activity in mesangial cells [Chardonnens et al., 1989; Egan et al., 1990]. In confirmation of these reports, PKC activity was increased upon addition of 1 μM AVP (Fig. 1). The time necessary for stimulation

Fig. 3. Immunoblots demonstrating PI3K-dependent PKC activation. Cells were treated with the selective PI3K inhibitor LY294002 (25 μM) for 2 h prior to exposure to AVP. PKC isotype activation was determined by staining with phospho-specific antibodies. Inhibition of the PI3K pathway inhibited phosphorylation of (**A**) PKCα, (**B**) PKCε, (**C**) PKCδ, and (**D**) PKCζ indicating dependence on PI3K activation. The anti-phospho-PKCζ antibody shown in this blot had been generously donated by Dr. Toker. This blot represents a 6% SDS–polyacrylamide gels (PAGE). The expression of total proteins was used as loading control. As before, bars represent the ratio of the intensity of the band obtained with the phospho-specific antibody (**upper panel**) to the intensity of the band obtained with the total antibody (**lower panel**). The numbers were normalized to controls, and the mean ± SE obtained from three individual experiments.


A
Fold increase
p-PKCα (S657)
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AG1478 - - - - + + + +
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p-PKCε (S729)
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C
Fold Increase
p-PKCδ (T505)
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Fig. 4.

of PKC activity by AVP varied between the different PKC isoforms. While peak AVP-stimulated PKCα and PKCε activity was observed after 15 min, peak stimulation in PKCδ activity was observed after 5 min of AVP treatment (not shown).

AVP Stimulates PKC Phosphorylation in Rat Glomerular Mesangial Cells

In order to determine the time course of activation of the different PKC isoforms in rat mesangial cells, we treated serum starved cells for different periods of time with 1 μM AVP (15 s to 1 h). PKC activation is regulated by phosphorylation [Dutil et al., 1994]. Unless inhibited by compounds that prevent DAG or Ca^{++} binding or membrane translocation of the kinase, phosphorylation of PKC is a very good indicator of the activation of the protein (reviewed in Newton, 2003). Hence, we determined the phosphorylation state of these PKC isoforms as an indicator of its activation in response to AVP stimulation. AVP induced transient phosphorylation of PKC isoforms α, δ, ε, and ζ (Fig. 2). Both PKCα and PKCε exhibited peak phosphorylation at 15 min post-stimulation (Fig. 2A,B). While PKCδ showed peak phosphorylation at Thr 505 at 15 s post-stimulation (Fig. 2C), the phosphorylation of PKCζ was maximum at 30 s post-stimulation (Fig. 2D).

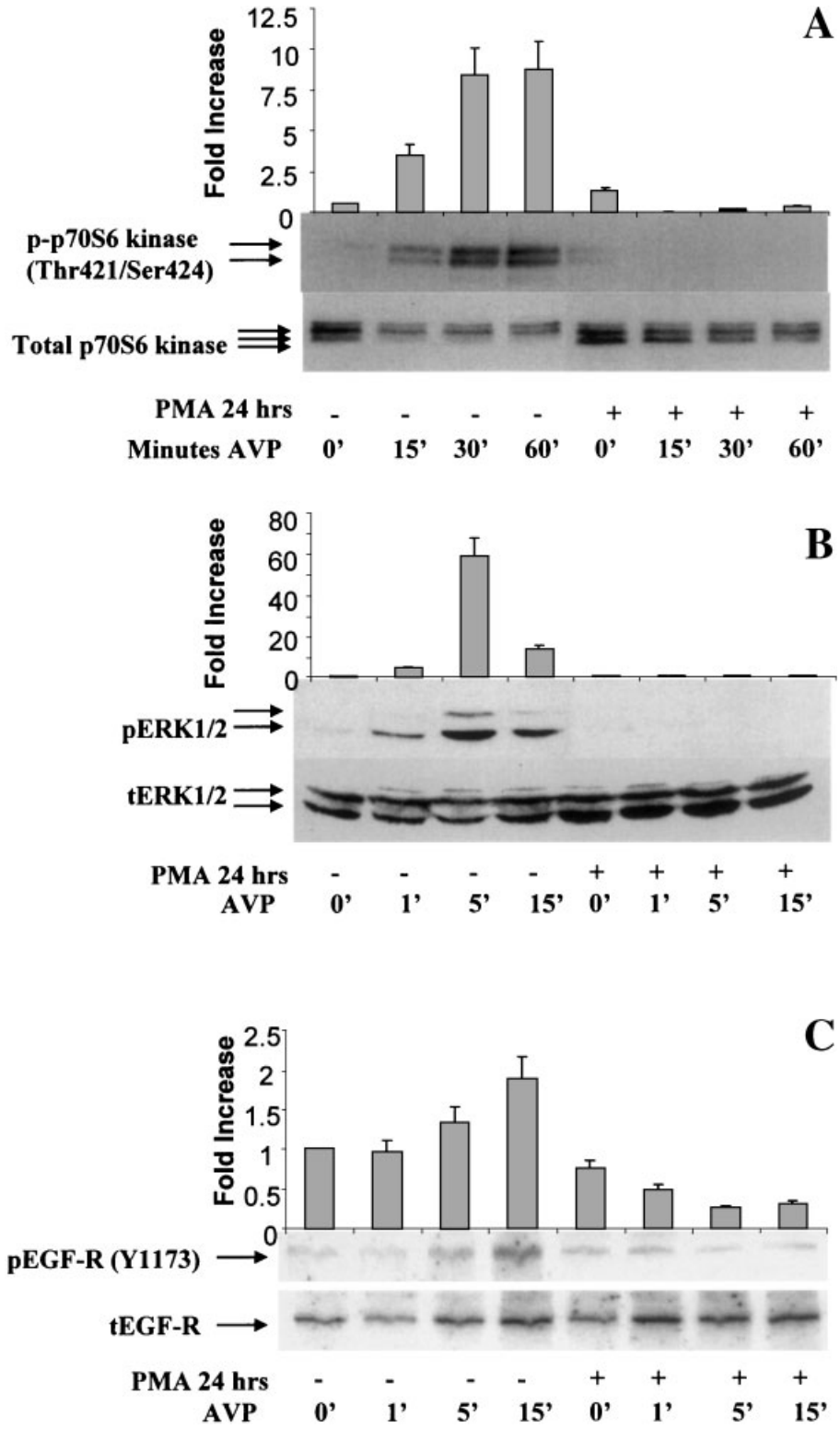
Phosphorylation of PKC Isoforms Is Dependent on EGF-R and PI3K Activation

Phosphatidylinositol 3,4,5-trisphosphate (PIP3), the lipid product of PI3K, was shown to activate all isoforms of PKCs [Dutil et al., 1994; Rupprecht et al., 1994; Toker et al., 1994; Chou et al., 1998]. Phosphorylation of all four isoforms of PKC (α, δ, ε, and ζ) in response to AVP was dependent on PI3K activation because phosphorylation was inhibited by pretreatment with the PI3K inhibitor LY 294002 (25 μM) (Fig. 3). This is a widely used specific inhibitor of PI3K that has not been shown to affect other signal transduction pathways [Vlahos et al., 1994]. We earlier demonstrated that AVP stimulated EGF-R phosphorylation in rat renal glomerular mesangial cells [Ghosh et al., 2001]. We, therefore, examined whether activation of the various PKC isoforms studied was downstream of AVP-stimulated EGF-R activation. Pretreatment with the EGF-R inhibitor AG 1478 (20 μM) also inhibited AVP-stimulated phosphorylation of all four PKC isoforms (Fig. 4). We previously demonstrated the specificity of AG1478 and showed that this concentration of AG1478 did not affect other RTKs but was necessary for complete inhibition of EGF-R activation in these cells [Ghosh et al., 2001].

AVP-Induced ERK1/2 and p70S6 Kinase Activation may be Mediated by PKC

We previously showed that AVP is a potent stimulator of p70S6 kinase and ERK in mesangial cells [Ghosh et al., 2001]. It is well established that the activation of ERK and p70S6 kinase is regulated by phosphorylation [Boulton et al., 1991; Dufner and Thomas, 1999]. To determine whether AVP-stimulated p70S6 kinase and ERK activation is mediated by PKC, we pretreated the cells overnight (chronic treatment) with 400 nM PMA prior to AVP stimulation as described by us earlier [Kreisberg et al., 1996]. Prolonged incubation with PMA downregulates novel and conventional PKC isoforms and inactivates AVP-stimulated PKC activation without affecting AVP receptor number [Troyer et al., 1992]. AVP-stimulated p70S6 kinase and ERK phosphorylation was inhibited by chronic (overnight) treatment with 400 nM PMA (Fig. 5A,B), indicating that p70S6 kinase and ERK activation was indeed mediated by PKC activation. In addition, overnight PMA treatment also inhibited the phosphorylation (activation) of the EGF-R at Tyr 1173 (Fig. 5C), indicating the

Fig. 4. Immunoblots demonstrating the effect of inhibition of EGF-R activation on phosphorylation of PKC isoforms in mesangial cells. Mesangial cells were pretreated with the tyrphostin-derived EGF-R antagonist AG1478 (20 μM) for 1 h prior to AVP treatment. **A**: AVP-stimulated increase in phosphorylation of PKCα was inhibited by AG1478 pretreatment, however, some basal phosphorylation level was retained. The blot was then stripped and restained with total PKCα antibody (**lower panel**) as loading control. This blot was run on a 10% SDS–PAGE. **B–D**: AVP-stimulated phosphorylation of PKCε, δ, and ζ was also inhibited by AG1478. As before, **lower panels** display the expression of the total proteins. A–C: The bars represent the mean (±SE) ratio of the intensity of the band obtained with the phospho-specific antibody (**upper panel**) to the intensity of the band obtained with the total antibody (lower panel), normalized to controls, from three individual experiments. D: Bars represent the mean ± SD from two experiments.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AVP 0' 1' 5' 15' 0' 1' 5' 15'

Fig. 5.

presence of a feedback loop between PKC and the EGF-R.

Rottlerin, but not a PKCε Translocation Inhibitor or Gö6976, Affects AVP Stimulated p70S6 Kinase Phosphorylation

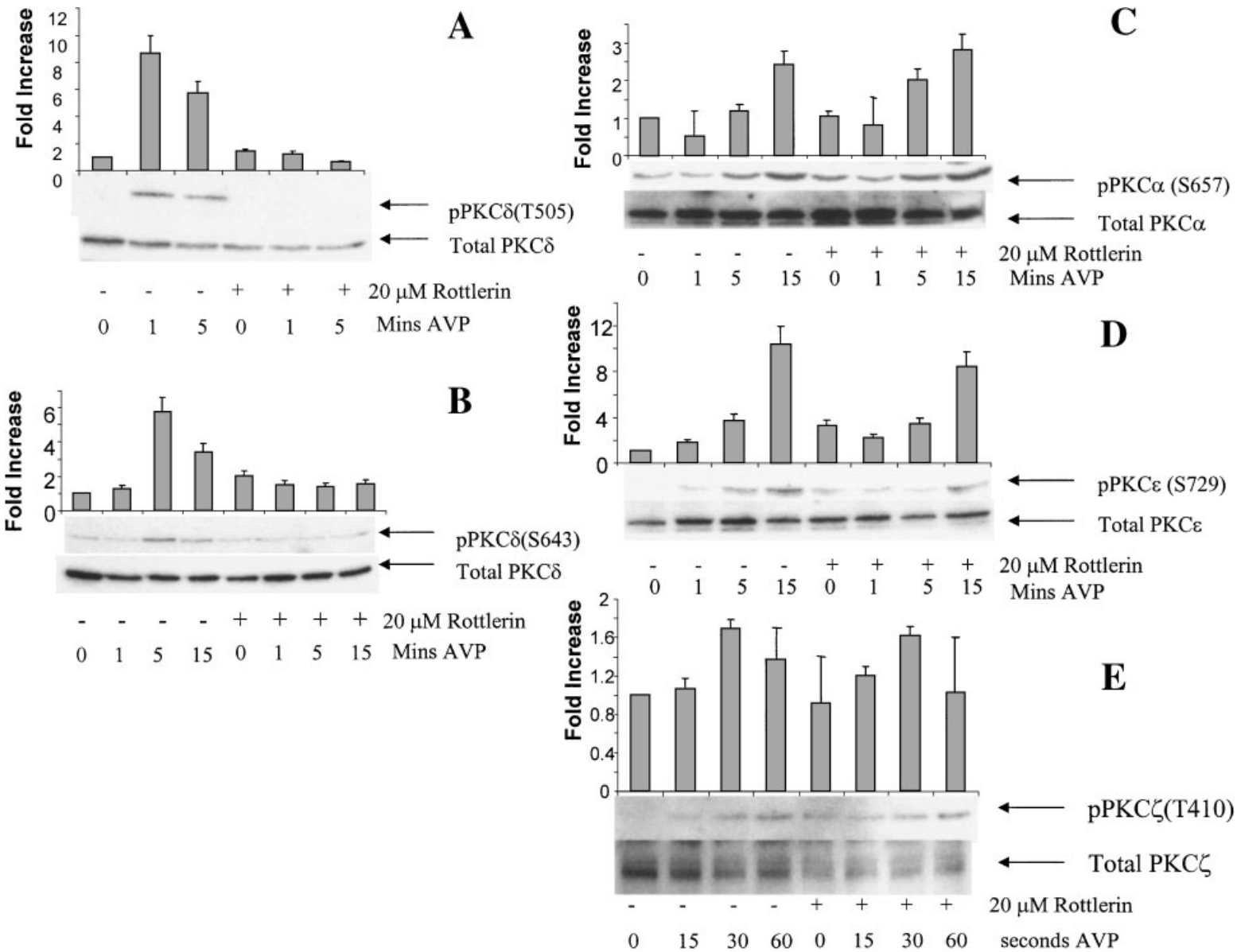
Since PMA affects all novel and conventional PKC isoforms, we next determined which PKC isoform mediated AVP's actions on p70S6 kinase and ERK1/2. For this purpose, we used Rottlerin, a specific inhibitor of PKCδ, a specific inhibitor of PKCε membrane translocation and, Gö6976, an inhibitor of conventional PKCs. Rottlerin inhibits the phosphorylation of PKCδ by competitively inhibiting ATP binding [Gschwendt et al., 1994]. Rottlerin inhibited AVP-stimulated phosphorylation of PKCδ (T505) and PKCδ (S643), but did not affect AVP-stimulated phosphorylation of PKCα, ε, and ζ isoforms (Fig. 6). It is notable that, unlike phosphorylation of PKCδ (T505), which is absent under unstimulated conditions, we observed baseline phosphorylation of PKCδ (S643). AVP stimulated transient phosphorylation of PKCδ (S643) in rat mesangial cells with a peak at 5 min. This phosphorylation was inhibited by 30 min pretreatment with Rottlerin. These data indicate the specificity of Rottlerin for PKCδ inhibition. Rottlerin caused diminished activation of ERK by AVP and completely inhibited AVP-stimulated p70S6 kinase activation (Fig. 7). Rottlerin's effect on ERK was greater on ERK1 (the upper band) than on ERK2 (lower band).

Unlike Rottlerin, the PKCε translocation inhibitor (PKCε-ti) and Gö6976, an inhibitor of conventional PKCs, do not affect phosphorylation of the PKC isoform, but inhibit steps following phosphorylation-membrane translocation in case of PKCε-ti [Johnson et al., 1996] and Ca^{++} interaction in the case of Gö6976 [Martiny-Baron et al., 1993]. Therefore, we determined the specificity of these compounds by determining their effect on PKC activity. The PKCε-ti inhibited the activation of PKCε, but not that of PKCα or PKCδ (Fig. 8A). Similarly, Gö6976 inhibited the activation of PKCα but not the activation of PKC isoforms δ and ε (Fig. 9A). These pharmacological agents had different effects on ERK1/2 phosphorylation. While PKCε-ti inhibited AVP-stimulated ERK1/2 phosphorylation (Fig. 8B), Gö6976 potentiated AVP's effects on ERK1/2 (Fig. 9B). In contrast to Rottlerin (Fig. 7B), PKCε-ti and Gö6976 failed to affect AVP-stimulated p70S6 kinase phosphorylation (Figs. 8C and 9C).

PKCζ Affects p70S6 Kinase but not ERK1/2 Phosphorylation Stimulated by AVP

In addition to the PKC isoforms studied above, we also studied the effect of the atypical PKC isoform, PKCζ, on AVP-stimulated ERK and p70S6 kinase activation. Rat mesangial cells were transfected with GFP (pEGFP-C1) expression vector alone, or co-transfected with GFP and dominant negative PKCζ (K281W), using lipofectAMINE PLUS (see Materials and Methods). Twenty-four hours after transfection, the cells were trypsinized and sorted by flow cytometry, and the GFP-expressing cells collected and plated in tissue culture dishes. Further flow cytometric analysis of the sorted cells showed that 96–98% of collected cells were GFP-expressing (not shown), indicating that only transfected cells were collected by sorting. Replated cells were treated with AVP (1 μM) for various times and collected in lysis buffer for analysis. Transfection of the dominant negative plasmid inhibited AVP-stimulated phosphorylation of PKCζ (Fig. 10A). Immunoblotting showed that transfection of dominant negative PKCζ did not inhibit AVP-stimulated ERK activation, but significantly delayed p70S6 kinase phosphorylation (Fig. 10B,C). These data indicate that PKCζ may play an important role in AVP-stimulated activation of p70S6 kinase but not that of ERK1/2.

Fig. 5. Immunoblots demonstrating the effect of 24 h of phorbol 12-myristate 13-acetate (PMA, 400 nM) on AVP-stimulated ERK1/2 and p70S6 kinase activation (phosphorylation). Mesangial cells were grown to confluence and serum starved for 48 h. PMA was added 24 h prior to AVP treatment (24 h after serum starvation), and cells stimulated with AVP for 0–60 min. PMA treatment for 24 h abolished activation of both ERK and p70S6 kinase by AVP. **A**: p70S6 kinase phosphorylation was determined by staining blots with a phospho-specific p70S6 kinase antibody [p-p70S6 kinase (T421/S424)]. **Lower panel** shows the total protein. **B**: ERK activation was assessed by staining with a phospho-specific ERK1/2 (T202/Y204) antibody (pERK1/2). **Lower panel** shows total ERK as control. **C**: EGF-R phosphorylation was assessed by staining with a phospho-specific EGF-R (Y1173) antibody (pEGF-R). **Lower panel** shows total EGF-R as control. Bars represent the normalized mean (±SE) ratio of the intensity of the band obtained with the phospho-specific antibody (**upper panel**) to the intensity of the band obtained with the total antibody (lower panel), from three individual experiments.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pPKCδ(T505)
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0.4
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pPKCζ(T410)
Total PKCζ
- - - - + + + + 20 μM Rottlerin
0 15 30 60 0 15 30 60 seconds AVP

Fig. 6.

AVP-Stimulated Mesangial Cell Proliferation Is Inhibited by Rottlerin

We next determined whether AVP-stimulated proliferation was dependent on PKC activation. Mesangial cells were treated with the inhibitors described above to determine the role of each PKC isoform in cell proliferation. Rottlerin was the most potent inhibitor of mesangial cell proliferation (66% inhibition) while the other inhibitors had much smaller effects ($\leq$10%) (Fig. 11). These data suggest that PKCδ plays an important role in mesangial cell proliferation.

DISCUSSIONS

We previously showed that AVP-stimulated rat renal glomerular mesangial cell proliferation was mediated by EGF-R transactivation [Ghosh et al., 2001]. In this paper, we extend our previous observations and show that PKC plays a prominent role in mediating events occurring downstream of AVP-stimulation in mesangial cells. AVP treatment for 0–60 min stimulated the serine/threonine phosphorylation of PKC isoforms α, δ, ε, and ζ. The activation of PKC isoforms was dependent on EGF-R and PI3K activation. Inhibition of specific PKC isoforms by pharmacological inhibitors or dominant negative cDNA differentially affected AVP-induced activation of ERK and p70S6 kinase, and cell proliferation.

The PI3K Signaling Pathway Affects PKC Activation

The two subunit PI3K molecule (regulatory p85 and catalytic p110) has been linked to cell growth and transformation, differentiation, motility, insulin action, and cell survival. PI3K appears to possess both lipid and protein kinase activities [Carpenter et al., 1993; Dhand et al., 1994]. Activation of PI3K occurs through tyrosine phosphorylation by RTKs (e.g., EGF-R) [Kapeller and Cantley, 1994; Rodriguez-Viciana et al., 1994]; activated, GTP-bound Ras also binds to and activates PI3K [Rodriguez-Viciana et al., 1994]. In this paper, we show that PI3K activation is necessary for AVP-stimulated PKC phosphorylation. It may be noted here that class IA PI3Ks are activated by RTKs, such as the EGF-R, while class IB PI3Ks are activated by GPCRs, such as the vasopressin V1 receptor [Selheim et al., 2000; Vivanco and Sawyers, 2002]. Both class IA and class IB PI3Ks are capable of activating PKCs. Therefore, AVP-induced PI3K activation, which stimulates PKC phosphorylation, may be direct, independent of EGF-R, or may be mediated by EGF-R.

Cross-Talk Between EGF-R and PKCs

Several lines of evidence indicate that the EGF-R both regulates, and is regulated by, PKC activation [Summers and Bass, 1997; Bao et al., 2000; Gao et al., 2001]. While there is considerable evidence showing PKC mediated EGF-R phosphorylation [Summers and Bass, 1997; Bao et al., 2000], PKC activation has also been reported to be downstream of EGF-R activation [Gao et al., 2001]. We have shown that in our cells, AVP-stimulated PKC activation is inhibited by the EGF-R inhibitor AG1478. At the same time, inhibition of conventional and novel PKC activation with chronic PMA treatment inhibited EGF-R phosphorylation at Y1173, which is a major autophosphorylation site on the EGF-R. Thus, there exists a feedback loop by which certain PKC isoforms activate EGF-R and are then further activated themselves as a result of EGF-R phosphorylation. PKCs likely mediate Pyk2 phosphorylation, which may be an intermediate step in AVP-induced EGF-R transactivation [Ghosh et al., 2001]. In turn, EGF-R may activate PI3K and PLCγ which further activates the PKCs.

PKC Dependent ERK and p70S6 Kinase Activation

AVP is a potent stimulator of ERK [Foschi et al., 1997; Ghosh et al., 2001]. While ERK activation is usually assumed to be downstream of Ras activation, there is also evidence showing that PKCs can stimulate Raf, and thereby ERK, independent of Ras activation [Ueda et al., 1996; Schonwasser et al., 1998]. In our cells, chronic PMA treatment to downregulate conventional

Fig. 6. Immunoblots with phospho-specific antibodies demonstrates the effect of Rottlerin on PKC isoform phosphorylation. **A**, **B**: Rottlerin inhibited AVP-stimulated phosphorylation of PKCδ (T505 and S643). **C**–**E**: In contrast, Rottlerin did not inhibit AVP-stimulated phosphorylation of PKCα (S657), PKCε (S729), or PKCζ (T410). Staining with antibodies to the total protein were used as loading control of proteins. Bars represent the normalized mean (±SE) ratio of the band intensity of the phospho-specific antibody (**upper panel**) to the band intensity of the total antibody (**lower panel**) from three individual experiments.

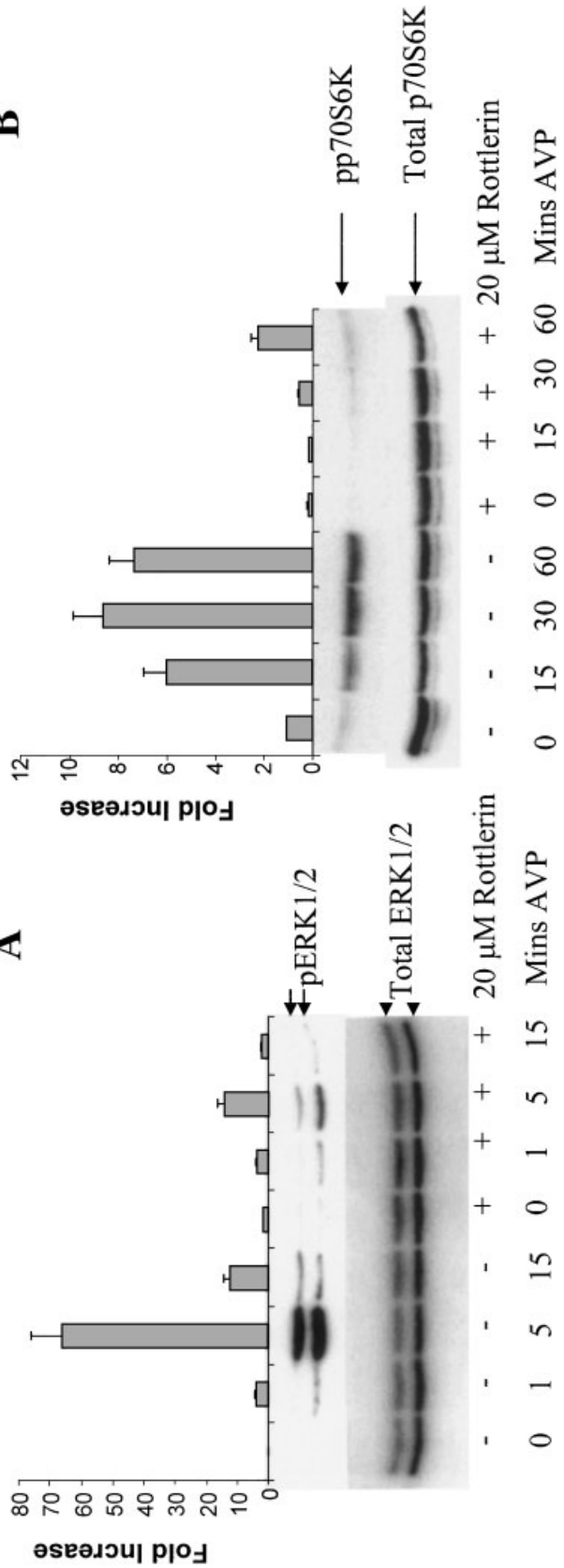


Fig. 7. Immunoblots with phospho-specific antibodies demonstrates the effect of Rottlerin on AVP-stimulated (**A**) ERK and (**B**) p70S6 kinase activation. Rottlerin inhibited AVP-stimulated p70S6 kinase phosphorylation and decreased phosphorylation of ERK. Staining with antibodies to total proteins was used as loading control. Bars represent the normalized mean (±SE) ratio of the band intensity of the **upper panel** to the band intensity of the **lower panel** from three individual experiments.

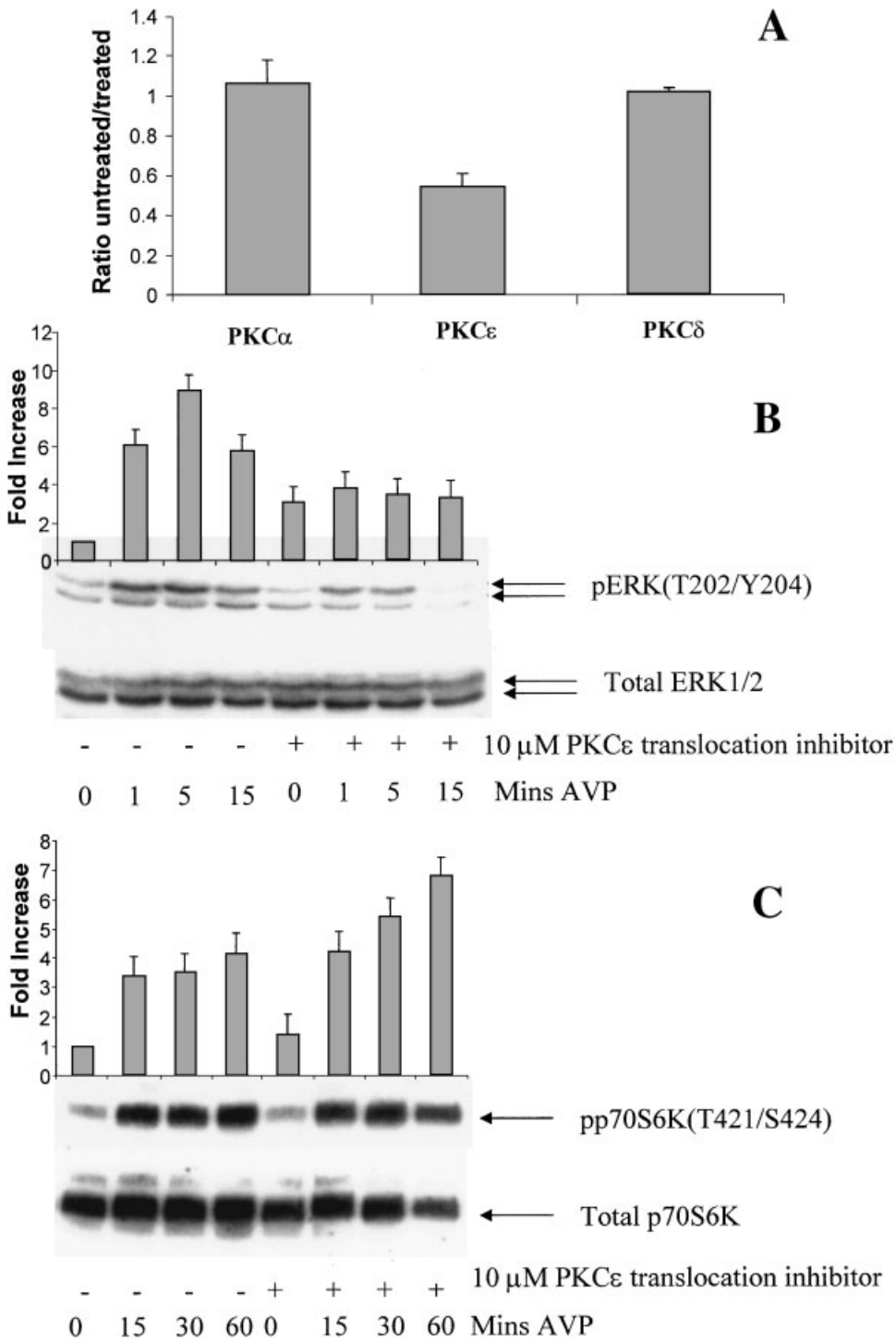


Fig. 8. **A**: PKC assay to determine specificity of the PKCε translocation inhibitor (PKCε-ti). Activities of PKCα, ε, and δ were determined in the presence or absence of the PKCε translocation inhibitor and are presented as the ratio of PKC activity in cells treated with PKCε translocation inhibitor to the PKC activity in untreated cells. Each bar represents the mean ± SD from two different experiments. **B**, **C**: Immunoblots with phospho-specific antibodies demonstrate the effect of PKCε-ti on AVP-stimulated ERK and p70S6 kinase activation. PKCε-ti decreased phosphorylation of ERK1/2 stimulated by AVP but did not inhibit AVP-stimulated p70S6 kinase phosphorylation. Staining with antibodies to total proteins was used as loading controls. Bars represent the normalized mean (±SE) ratio of the band intensity of the phospho-specific antibody to the band intensity of the total antibody from three individual experiments.

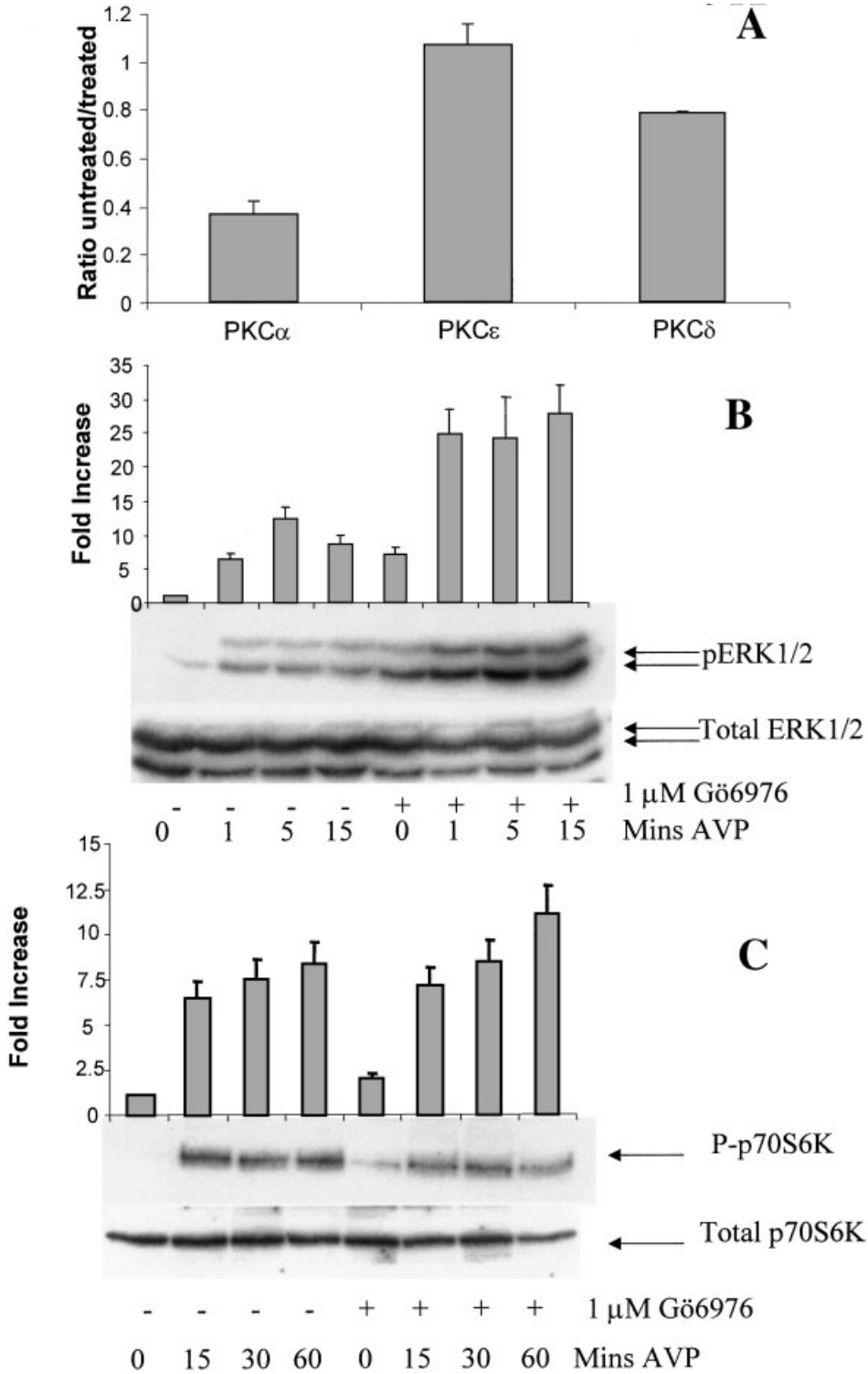


Fig. 9. **A**: PKC assay to determine specificity of the Gö6976. Activities of PKCα, ε, and δ were determined in the presence or absence of Gö6976 and are presented as the ratio of PKC activity in cells treated with Gö6976 to the PKC activity in untreated cells. Each bar represents the mean ± SD from two different experiments. **B**, **C**: Immunoblots with phospho-specific antibodies demonstrate the effect of Gö6976 on AVP-stimulated ERK and p70S6 kinase activation. Gö6976 potentiated AVP-stimulated phosphorylation of ERK, but did not affect AVP-stimulated p70S6 kinase phosphorylation. Staining with antibodies to total proteins were used for loading controls. Bars represent the normalized mean (±SE) ratio of the phospho-specific antibody band intensity to the total antibody band intensity from three individual experiments.

and novel PKC isoforms inhibited AVP-stimulated ERK and p70S6 kinase phosphorylation. These results are consistent with the observations of other investigators. AVP-induced activation of MAPK in vascular smooth muscle cells was regulated by PKC activation [Kribben et al., 1993]. Acute PMA stimulation of p70S6 kinase has been reported in corneal epithelial cells [Chandrasekher et al., 2001] while chronic PMA treatment inhibited p70S6 kinase activation in Swiss 3T3 cells [Kim et al., 2000].

The various PKC isoforms had different effects on ERK and p70S6 kinase phosphorylation. Both novel PKC isoforms present in mesangial cells (PKCδ and PKCε) affected ERK1/2 phosphorylation. Rottlerin, an inhibitor of PKCδ and a PKCε translocation inhibitor both prevented AVP-stimulated ERK1/2 phosphorylation. In contrast, Gö6976, an inhibitor of conventional PKCs, stimulated ERK1/2 phosphorylation in mesangial cells, suggesting an inhibitory effect of PKCα on AVP-stimulated ERK1/2 phosphorylation. AVP-stimulated ERK1/2 phosphorylation was not affected by inhibition of PKCζ using a dominant negative PKCζ cDNA.

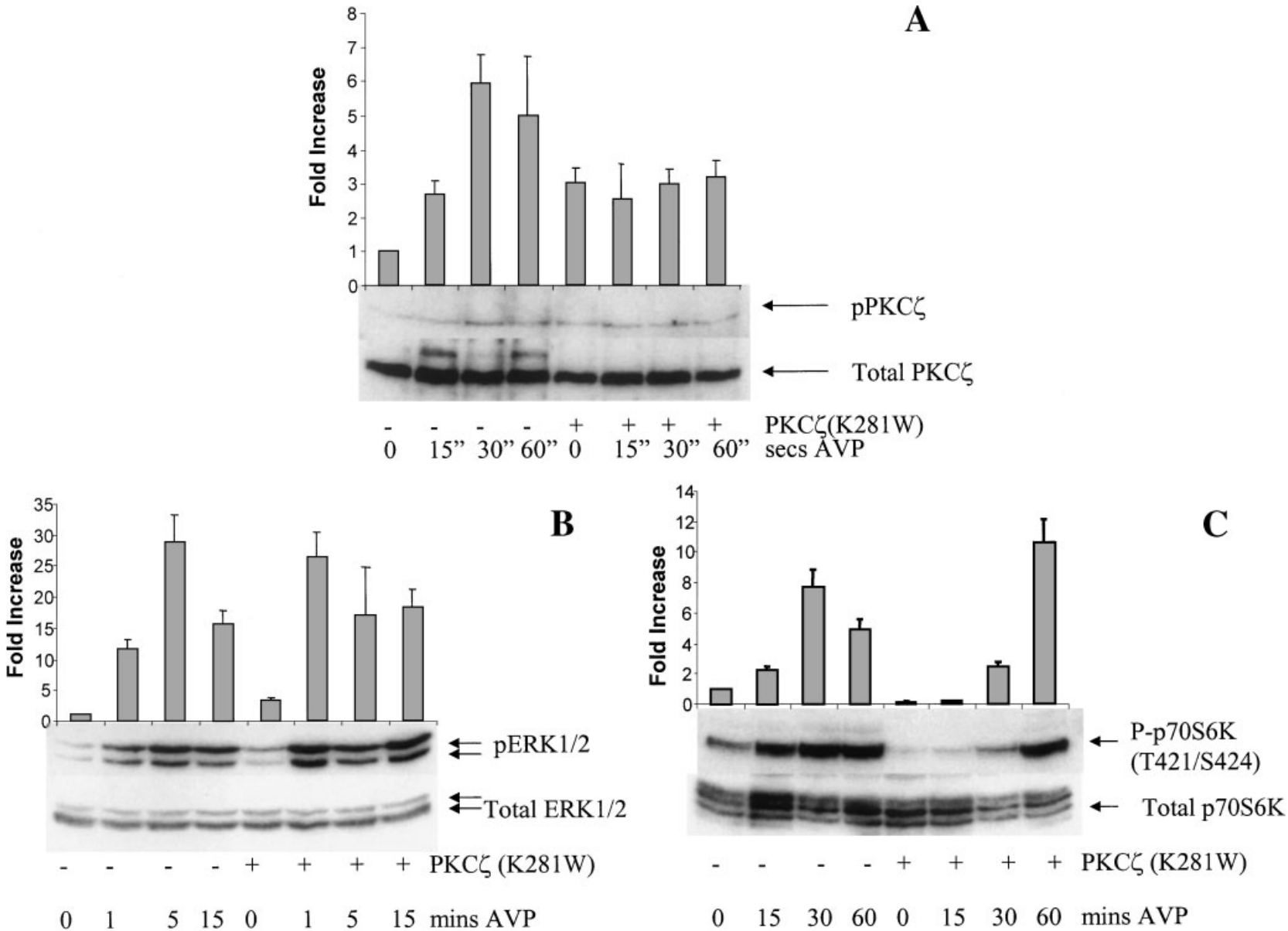
In contrast to ERK, AVP-stimulated p70S6 kinase phosphorylation was inhibited only by Rottlerin, while inhibition of PKCζ using the dominant negative construct delayed, but did not completely prevent, AVP-stimulated p70S6 kinase phosphorylation. Neither Gö6976, nor the PKCε translocation inhibitor, affected AVP-stimulated p70S6 kinase phosphorylation. PKCζ, which can be activated by mitogenic stimulation downstream of PI3K [Akimoto et al., 1996] has received considerable attention as an activator of p70S6 kinase [Romanelli et al., 1999]. Our data show that PKCδ can also regulate p70S6 kinase phosphorylation.

AVP-Stimulated Mesangial Cell Proliferation may be Mediated by PKCδ

The same inhibitors also had differential effects on AVP-stimulated mesangial cell proliferation. Only Rottlerin had a significant effect on proliferation, indicating a major role of PKCδ in mesangial cell growth. This effect of Rottlerin was probably due to its effect on p70S6 kinase. We showed earlier that p70S6 kinase had a much larger effect on mesangial cell proliferation compared to ERK [Ghosh et al., 2001]. p70S6 kinase, a downstream effector of PI3K, is an important regulator of cell growth. It participates in the translation of mRNAs, which contain an oligopyrimidine tract at their transcriptional start site. The regulation of p70S6 kinase includes phosphorylation at multiple sites. PI3K signaling through p70S6 kinase acts synergistically with Ras/MAPK to stimulate the G_1 to S phase transition of the cell cycle [Cantley et al., 1991; Rodriguez-Viciana et al., 1994; Klippel et al., 1998]. p70S6 kinase is also phosphorylated by a phosphatidylinositol kinase-related kinase, mTOR (mammalian target of rapamycin) [Alessi et al., 1998; Dufner and Thomas, 1999; Grewe et al., 1999]. The lack of an effect of the other inhibitors, PKCε-ti and Gö6976 on mesangial cell proliferation probably stems from their inability to affect p70S6 kinase in these cells.

Vasopressin stimulates mesangial cell contraction, which contributes to its effect on the glomerular microcirculation as well as stimulates mesangial cell growth [Ganz et al., 1988; Ghosh et al., 2001]. Although much is known about the mechanisms of its vasopressor activity, little is known about the mechanisms involved in stimulating cell proliferation. Our previous observations showed that vasopressin exerted its effect on mesangial cell proliferation by transactivating the EGF-R via the activation of c-Src which resulted in activation of both the MAPK and PI3K signaling pathways [Ghosh et al., 2001]. Here we extended these findings and report that PKCδ played a major role in AVP-stimulated ERK and p70S6 kinase phosphorylation and cell proliferation. Different PKC isoforms, however, affected ERK and p70S6 kinase differently, thereby

Fig. 10. (*Overleaf*) Inhibition of PKCζ with a dominant negative plasmid, pCMV5-PKCζ (K281W). pCMV5-PKCζ (K281W), and pEGFP-C1 were transiently co-transfected into rat mesangial cells using lipofectAMINE. Transfected cells expressing GFP were sorted by flow cytometry. **A**: Immunoblot with phospho-PKCζ antibody showing transfected cells exhibited inhibition of AVP-stimulated PKCζ phosphorylation. Staining with antibody total PKCζ determined protein loading. Bars represent the normalized mean (±SE) ratio of the phospho-specific antibody band to the total antibody band from three individual experiments. **B**, **C**: Immunoblots shows that transient transfection of PKCζ(K281W) (B) had no effect on ERK phosphorylation but (C) delayed considerably, AVP-stimulated p70S6 kinase phosphorylation. Bars represent the normalized mean (±SE) ratio of the phospho-specific antibody to the total antibody from three individual experiments.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B
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pERK1/2
Total ERK1/2
- - - - + + + + PKCζ (K281W)
0 1 5 15 0 1 5 15 mins AVP
C
Fold Increase
P-p70S6K
(T421/S424)
Total p70S6K
- - - - + + + + PKCζ (K281W)
0 15 30 60 0 15 30 60 mins AVP

Fig. 10.

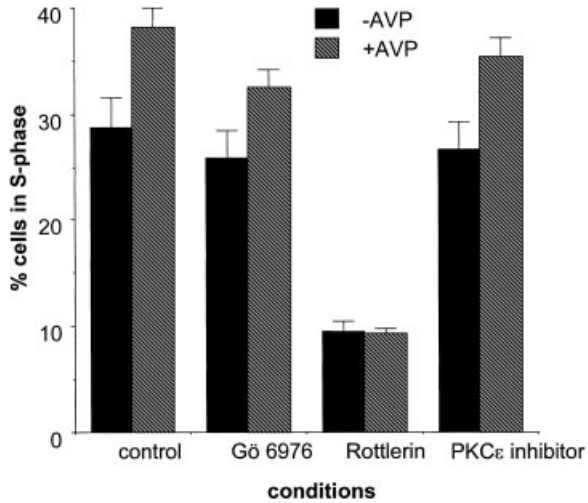

Fig. 11. Flow cytometric analysis of the effect of isoform specific PKC inhibitors on the percentage of control and AVP-treated cells in S phase of the cell cycle. These data represent the mean ± SD of two independent experiments.

having various effects on proliferation as well. Thus, increased secretion of vasopressin, as measured in patients with diabetic nephropathy [Nishikawa et al., 1996], may stimulate mesangial cell proliferation and alter the glomerular microcirculation.

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