$PGF_{2\alpha}$-Induced Signaling Events in Glomerular Mesangial Cells (44005)

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Abstract. **Of the various arachidonate cyclooxygenation eicosanoids synthesized in the normal and injured renal glomerular capillary, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the most abundant and potent in eliciting signaling events and biologic responses including contraction and proliferation of glomerular capillary pericytes known as mesangial cells. The regulation of $PGF_{2\alpha}$-induced signaling in these cells is unknown. The present studies assessed two key signaling events in response to $PGF_{2\alpha}$ in mesangial cells; activation of phospholipase C (PLC) and protein kinase C (PKC). Mechanisms regulating PLC activation were also explored. Incubation of cultured growth arrested rat mesangial cells with $PGF_{2\alpha}$ (1 μM) resulted in activation of a phosphatidyl inositol–specific phospholipase C (PI-PLC) assessed as increased generation of polyphosphates in myo-[^{3}H]-inositol–labeled cells and as increased diacylglycerol (DAG) mass levels measured by a radioenzymatic assay. Generation of both inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate occurred, the former constituting 70% of total inositol trisphosphates. Enhanced generation of inositol 1,4-bisphosphate (IP_2) also occurred and was greater than that of inositol 1,4,5-trisphosphate (IP_3), indicating that PI-PLC utilized the phosphatidyl inositol monophosphate (PIP) to a greater extent than the phosphatidyl inositol bisphosphate (PIP_2) substrate. Generation of DAG in response to $PGF_{2\alpha}$ occurred in a biphasic pattern characterized by an early transient rise that peaked concomitantly with IP_3 at 15 sec, and a late sustained increase at 2, 5, and 15 min that was not associated with an increase in IP_3. $PGF_{2\alpha}$ also activated PKC assessed as translocation of enzyme activity from cytosolic to membrane fractions. Inhibition of PKC using H-7 enhanced $PGF_{2\alpha}$-induced generation of IP_3 at 15 sec but attenuated generation of DAG at 15 min. A more selective PKC inhibitor, Calphostin C, dose-dependently increased basal IP_3 generation and also attenuated generation of DAG in response to $PGF_{2\alpha}$. This indicates that PKC negatively modulates $PGF_{2\alpha}$-induced PI-PLC activation, and that the late sustained DAG generation in response to $PGF_{2\alpha}$ is regulated by a PKC-dependent phospholipase other than PLC. The mechanisms of PI-PLC stimulation in response to $PGF_{2\alpha}$ were further explored using inhibitors of protein tyrosine phosphorylation and of guanine nucleotide–binding (G) protein activation. Inhibition of protein tyrosine phosphorylation using genistein had no effect on IP_3 or DAG generation. ADP ribosylation of Gi using pertussis toxin (PTx) had no effect on IP_3 generation in response to $PGF_{2\alpha}$. The inhibitor of receptor-coupled PI-PLC activation aminosteroid compound U-73122 that blocks G_{PLC} was also ineffective.**

The observations indicate that $PGF_{2\alpha}$ stimulates a PI-PLC which is under negative feedback regulatory control by PKC, and a phospholipase other than PLC which is under positive regulatory control by PKC. $PGF_{2\alpha}$-induced PI-PLC activation is independent of protein tyrosine phosphorylation and of PTx-sensitive G proteins.

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Of the various arachidonate cyclooxygenation eicosanoids synthesized by the renal glomerulus and its various intrinsic cells, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the most abundant. This has been demonstrated in isolated human and rat glomeruli, and in cultured glomerular cells (epithelial, mesangial) (1). The glomerular synthesis of $PGF_{2\alpha}$ is increased following injury, as was shown following antibody-mediated injury directed against the glomerular basement mem-

brane, epithelial or mesangial cells (2). Once released, $PGF_{2\alpha}$ may act in a paracrine or autocrine manner on intrinsic cells of the glomerular capillary to produce biologic responses. These have best been characterized in cultured mesangial cells which are specialized pericytes of the glomerular capillary that contract and proliferate in response to $PGF_{2\alpha}$ (3). Although $PGF_{2\alpha}$ is a vasoactive and mitogenic eicosanoid, the extent to which it mediates constriction of the glomerular capillary and proliferation of intrinsic glomerular cells following injury is difficult to determine. This is because, following the discovery of thromboxane A_2 (TxA_2) and the leukotrienes, and the demonstration that inhibition of the synthesis or antagonism of the receptor of these eicosanoids ameliorates the hemodynamic manifestations of various forms of glomerular injury (4), the interest in $PGF_{2\alpha}$ has declined. Moreover, selective inhibitors of $PGF_{2\alpha}$ synthesis or antagonists of its receptor are not available to address these questions definitively. Nonetheless, it is clear that, in addition to being the most abundant glomerular eicosanoid, $PGF_{2\alpha}$ produces potent biologic effects on glomerular cells the magnitude of which exceeds that of TxA_2 and the leukotrienes. We have demonstrated that, compared with TxA_2 mimetics and leukotriene (LT) D_4, $PGF_{2\alpha}$ is the most potent in activating phosphatidyl inositol–specific phospholipase C (PI-PLC) and protein kinase C and in increasing DNA synthesis in cultured glomerular mesangial cells (5). With respect to its mitogenic potential, the potency of $PGF_{2\alpha}$ is comparable to that of epidermal growth factor in stimulating phosphorylation of the 40S ribosomal subunit S6 and in activating protein and DNA synthesis (6). These observations prompted us to characterize $PGF_{2\alpha}$-induced signaling events and assess their regulation in cultured mesangial cells. The present studies assessed the effect of $PGF_{2\alpha}$ on two signaling events, activation of PLC and protein kinase C (PKC), and explored mechanisms regulating PLC activation.

Materials and Methods

Mesangial Cell Cultures. Rat mesangial cells originating from glomerular explants were cultured using established methods. Briefly, glomeruli were isolated by sequential sieving of minced kidneys from 80- to 100-g Sprague-Dawley rats, digested for 15 min with 0.1% type IV collagenase (460 units/ng; Sigma Chemical Co., St. Louis, MO) and incubated in RPMI-1640 medium containing 15% fetal calf serum (FCS), 0.66 units/ml of insulin, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 250 ng/ml of amphotericin B. Glomerular outgrowths were maintained at 37°C in a 5% CO_2 humidified atmosphere and yielded pure lines of mesangial cells within 6 weeks. Mesangial cells were characterized by their typical stellate or spindle-shaped morphology noted using phase-contrast microscopy and by staining for the presence of Thy 1 antigen (7). Independent mesangial cell lines were subcultured every 2 weeks and were used in passages 10–30.

Assessment of Phospholipase C Activation. Phospholipase C activation was assessed as generation of (i) the inositol polyphosphates (IP) (inositol 1,4,5-trisphosphate, inositol 1,3,4-trisphosphate, and inositol 1,4-bisphosphate) and (ii) diacylglycerol (DAG), measured as total cell DAG mass.

To measure inositol polyphosphates, mesangial cells grown in 60-mm culture dishes were incubated in a low inositol medium containing 50% Dulbecco's modified Eagle's medium, 50% HAMS F-10, 15% FCS, antibiotics, and insulin. When the monolayers were confluent, cells were labeled for 36 hr with 2 μCi/ml of myo-2[^{3}H]-inositol (20 Ci/mmol, Amersham) in serum-free medium containing insulin and antibiotics. Plates were washed with Krebs-Ringer solution containing 118 m*M* NaCl, 4.6 m*M* KCl, 24.9 m*M* $NaHCO_3$, 1 m*M* KH_2PO_4, 11.1 m*M* glucose, 1.1 m*M* $MgSO_4$, 1.0 m*M* $CaCl_2$, 5 m*M* HEPES, and 0.1% bovine serum albumin, pH 7.4. $PGF_{2\alpha}$ (Biomol, Plymouth Meeting, PA) dissolved in ethanol was diluted to 1 μ*M* in 2 ml of the Krebs-Ringer solution (the final concentration of ethanol <0.1%) and introduced in the cells for defined time periods (15–900 sec). $PGF_{2\alpha}$ was employed at 1 μ*M* since dose-response experiments have shown that this concentration maximally stimulates PLC (8). In experiments in which accumulation of phosphoinositides was measured more than 60 sec after the addition of $PGF_{2\alpha}$, LiCl (10 m*M*) was added to the incubation medium in order to prevent breakdown of IP_1. At defined time points following addition of $PGF_{2\alpha}$ to mesangial cells, the reaction was terminated by cold 10% TCA and plates were placed on ice for 30 min. The supernatant was subsequently removed and cells were washed with 1 ml of water, which was combined with the supernatant. Extraction of phosphoinositides was performed using three 5-ml washes with water-saturated ether. The aqueous phase was separated by centrifugation and the remaining ether allowed to evaporate. To separate the [^{3}H]-inositol phosphates present in the aqueous phase, a high-pressure liquid chromatography method was used as described in detail elsewhere (9). Briefly, elutions employed a linear gradient of 0.01 *M* $NH_4H_2PO_4$ to 1.75 *M* $NH_4H_2PO_4$, pH 3.8, for 30 min at a flow rate of 1 ml/min through a partisil SAX 10 μm anion exchange column (250 × 0.6 mm, Whatman, Clifton, NJ). This system provided good separations between the phosphoinositides 1,4-IP_2, 1,3,4-IP_3 and 1,4,5-IP_3. The results were expressed as percentage of cpm eluted as 1,4-IP_2, 1,4,5-IP_3, or 1,3,4 IP_3 compared with the respective cpm eluted from simultaneous vehicle treated controls.

DAG was measured in growth-arrested mesangial cells at defined time points (0.5–10 min) following incubation with $PGF_{2\alpha}$ introduced into cultures in a manner analogous to the one described above for the determination of changes in phosphoinositide generation. DAG mass was quantified by a radioenzymatic assay which employed *Escherichia coli* DAG kinase to quantitatively convert DAG present in cell lipid extracts to [^{32}P]–phosphatidic acid in the presence of [^{32}P]ATP (10). Upon completion of incubations with $PGF_{2\alpha}$, media was removed and 1 ml of ice-cold methanol was immediately added, cells were scraped, and 1 ml of chloroform was added. Lipids were subsequently extracted according to the method of Bligh and Dyer (11) except 1 *M* NaCl was used instead of water. Two aliquots, each containing one-fourth of the final organic phase, were dried under nitrogen and used for quantitation of DAG and determination of total cell lipid phosphorus (12). To measure DAG, the designated aliquots were brought to dryness and 20 μl of a carrier solution containing 7.5% octyl-β-D-glycoside, 5 m*M* cardiolipin in 1 m*M* diethylene triaminepentacetic acid was added to dissolve and carry DAG. Following a 15-sec sonication, 10 μl of DAG kinase (20 milliunits, Amersham, Arlington Heights, IL) was added followed by addition of 50 μl of a reaction buffer (100 m*M* imidazole hydrochloride, pH 6.6, 100 m*M* NaCl, 25 m*M* $MgCl_2$, 2 m*M* EDTA), 10 μl of 20 m*M* dithiothreitol and 10 μl of 10 m*M* [γ-^{32}P] ATP (2.5×10^5 cpm/nmol). The reactions were allowed to proceed at 25°C for 30 min and were stopped by the addition of 250 μl of methanol and 125 μl chloroform. Lipids were extracted as described above. Fifty microliters of the chloroform phase were subsequently spotted on Silica Gel 60 aluminum TLC plates (Merck, Rahway, NJ) and developed in chloroform: acetone:methanol:acetic acid:water (10:4:3:2:1, v/v). Areas on the TLC plates corresponding to [^{32}P]–phosphatidic acid spots were cut out and placed in scintillation vials for determination of radioactivity. A standard curve consisted of 1,2-diolein (32–1000 pmol range) substrate standards which were subjected to identical reactions and separations as the mesangial cell–derived DAG. The results were expressed as DAG mass in pmol/nmol mesangial cell lipid phosphorus.

Measurement of Protein Kinase C Activity. Protein kinase C (PKC) activity was measured in the cytosolic and membrane fractions of growth-arrested mesangial cells grown to confluence in 60-mm plates 15 min after the addition of $PGF_{2\alpha}$ or vehicle. Upon termination of incubations, cells were washed in buffer A, consisting of 50 m*M* Tris-HCl, 5 m*M* EDTA, 10 m*M* EGTA, 0.3% w/v 2-mercaptoethanol, 10 m*M* benzamidine, 50 μg/ml phenylmethyl sulfonyl fluoride and 50 μg/ml leupeptin. The suspension was then sonicated once for 3 sec at 40 watts and centrifuged (1000 rpm) for 10 min at 4°C to remove undissolved particles. The supernatant was further centrifuged for 30 min at 100,000*g* at 4°C to separate cytosol from membranes. The supernatant (cytosol) and the pellet (membranes) were resuspended in buffer A. Total protein in aliquots of cytosolic and membrane fractions was determined by a colorimetric method. To measure PKC activity, 25-μl aliquots of cytosolic and membrane fractions containing defined amounts of protein (0.5–5 μg) were mixed with 25 μl of a solution consisting of 12 m*M* calcium acetate, 8 mole% of detergent-dispersed phosphatidyl-*L*-serine, 24 μg/ml phorbol 12-myristate 13-acetate, 900 μ*M* of a phosphoacceptor peptide (amino acid sequence of N terminus: Arg Lys Arg Thr Leu Arg Arg Leu) (Amersham) (13), and 30 m*M* dithiothreitol in 50 m*M* Tris/HCl, pH 7.5. Following vortexing, 25 μl of a solution containing 150 μ*M* ATP, 45 m*M* magnesium acetate, and 0.2 μCi of [γ-^{32}P]ATP in 50 m*M* Tris/HCl was added. The reactants were incubated at 25°C for 15 min and the reaction was stopped by the addition of 100 μl of an acidic reaction quenching reagent (Amersham). One hundred twenty-five microliters of the reaction mixture was subsequently blotted on to a 2.5 × 2.5 cm peptide binding phosphocellulose paper (Whatman) which was dried and washed twice in a bath of 75 m*M* orthophosphoric acid solution for 10 min under gentle agitation. Following drying, the phosphocellulose papers were placed in a liquid scintillation vial in the presence of 10 ml of scintillant (Hydrofluor, Amersham) for β-radiation counting.

Results (cpm) were converted to specific radioactivity (*R*) on the basis of the following formula:

$$R(\text{cpm/nmol}) = \frac{\text{cpm per 10 } \mu\text{l magnesium [}^{32}\text{P]ATP}}{1.5},$$

where 10μl of 150 μ*M* ATP contains 1.5 nmol.

The total phosphate (*T*) transferred to the phosphoacceptor peptide was calculated by the formula

$$T = (\text{sample cpm} \times 1.4) - \text{blank cpm},$$

where 1.4 is a factor derived from the total assay volume (175 μl) divided by the volume applied to the binding paper (125 μl).

The pmoles of phosphate (*P*) transferred to the peptide per minute (PKC activity) was calculated by the formula

$$P = \frac{T \times 10^3 \text{pmol/min}}{I \times R},$$

where *I* is incubation time. The PKC activity derived was compared with that determined in reaction mixtures in which calcium acetate was replaced by 10 m*M* EGTA and phosphatidyl serine was omitted. Enzyme

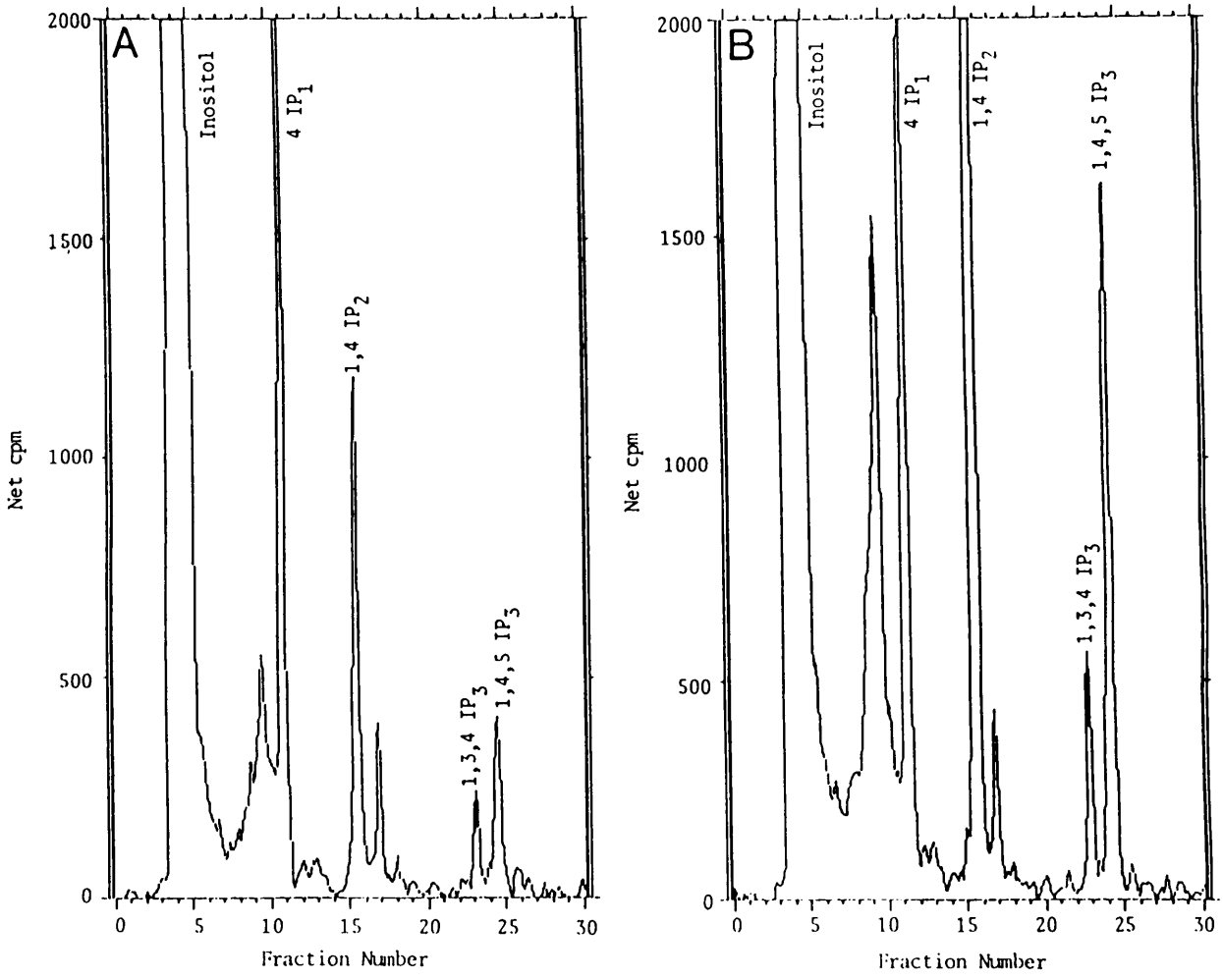

Figure 1. High-pressure liquid chromatograms of inositol polyphosphate generation in [^{3}H]-myoinositol–labeled mesangial cells in response to a 15-sec exposure to vehicle (A) or 1 μM $PGF_{2\alpha}$ (B). $PGF_{2\alpha}$ increased generation of 1,4,5-IP_3, 1,3,4-IP_3, and 1,4-IP_2.

activity values were factored by cytosolic or membrane protein concentration.

Regulation of $PGF_{2\alpha}$-Induced Signaling. These studies explored whether $PGF_{2\alpha}$-induced generation of inositol polyphosphates and DAG was dependent on protein kinase C, protein tyrosine phosphorylation, or G protein activation.

Generation of 1,4,5-IP_3, 1,3,4-IP_3, 1,4,-IP_2, and DAG was assessed in the presence and absence of two protein kinase C inhibitors, H-7 (1-[5-isoquinolinsulfonyl]-2-methylpiperazine dihydrochloride) (14) and Calphostin C. H-7 dissolved in DMSO was introduced as a final concentration of 50–200 μM in growth-arrested mesangial cells 3 hr prior to the addition of $PGF_{2\alpha}$. IP_3 and DAG were measured at 15 sec and 15 min, respectively. Calphostin C was dissolved in DMSO and introduced as final concentration of 0.01 to 0.5 μM. Preincubations with this PKC inhibitor were also 3 hr in duration but under fluorescent light as inhibition of PKC by Calphostin C is light dependent (15).

To explore a protein tyrosine phosphorylation-dependent mechanism of PLC activation in response to $PGF_{2\alpha}$, 1,4,5-IP_3 generation was assessed in the presence and absence of the tyrosine phosphorylation inhibitor genistein (16). This inhibitor, dissolved in DMSO (final volume DMSO: <0.1%), was introduced in growth-arrested mesangial cells at 100 μM 30 min prior to the addition of $PGF_{2\alpha}$. IP_3 and DAG generation were measured at 15 sec and 15 min, respectively.

To explore a G protein–dependent mechanism of $PGF_{2\alpha}$-induced PI-PLC activation, 1,4,5-IP_3 generation was assessed in the presence of pertussis toxin (PTx) or an inhibitor of receptor coupled G_{PLC}, the aminosteroid compound U-73122 (17). Mesangial cells were incubated with pertussis toxin (n = 6) at 100 ng/ml for 18 hr prior to the addition of $PGF_{2\alpha}$, and 1,4,5-IP_3 was measured at 15 sec. This dose of pertussis toxin has been shown to inhibit angiotensin II (Ang II)–induced IP_3 generation in cultured mesangial cells (18). U-73122, dissolved in DMSO, was incubated with mesangial cells (n = 12) at 20–50 μM for 18 hr prior to the addition of $PGF_{2\alpha}$ (final concentration of DMSO: <0.5%).

In all experiments outlined above results were expressed as mean ± SEM and were compared using

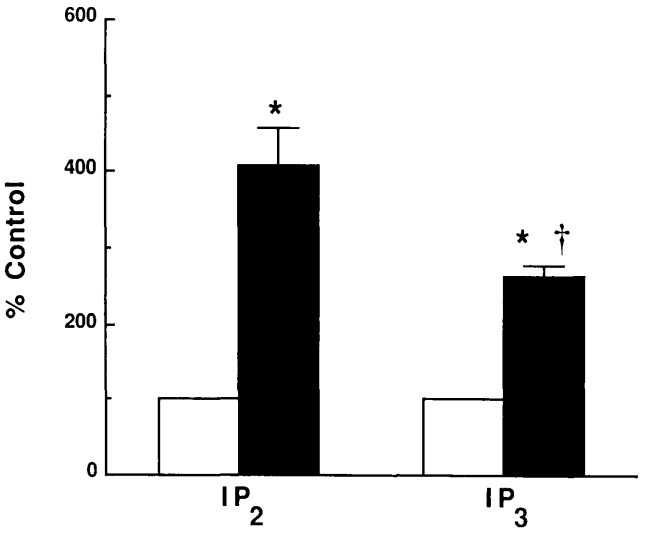

Figure 2. Effect of 15-sec stimulation with 1 μM $PGF_{2\alpha}$ on 1,4-IP_2 and 1,4,5-IP_3 generation in [^{3}H]-myoinositol labeled mesangial cells. $PGF_{2\alpha}$ (solid bars, n = 56, measurements derived from 25 separate experiments done in duplicate or triplicate) enhanced generation of both phosphoinositides compared with vehicle-treated controls (open bars, n = 53). The increment in 1,4-IP_2 was significantly greater than that in 1,4,5-IP_3. *$P < 0.05$ compared with vehicle-treated controls. †$P < 0.05$ compared with IP_2 generation in $PGF_{2\alpha}$-treated cells.

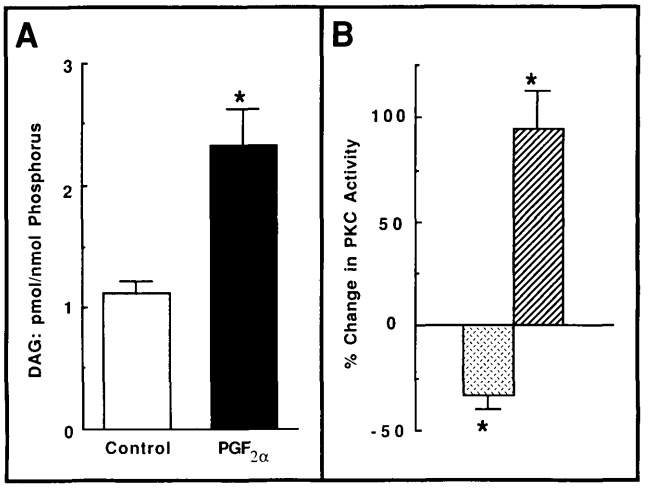

Figure 3. Effect of 1 μM $PGF_{2\alpha}$ on DAG mass levels in cultured mesangial cells (A) and on changes in protein kinase C activity (B) measured in the membrane (striped bar) and cytosolic fraction (stippled bar) of cultured mesangial cells. $PGF_{2\alpha}$ significantly increased DAG mass levels. There were significant decrements in cytosolic PKC activity with concomitant increments in membrane PKC activity (translocation) in response to $PGF_{2\alpha}$. Data is expressed as mean ± SEM for n = 4–6 observations. *$P < 0.05$ compared with vehicle-treated controls.

unpaired t test statistics. Generation of inositol polyphosphates was assessed in duplicate. DAG synthesis and PKC activity was assessed in quadruplicate.

Results

Effect of $PGF_{2\alpha}$ on PLC and PKC Activation. The effect of 1 μM $PGF_{2\alpha}$ on phosphoinositide generation assessed by HPLC of extracts of [^{3}H]-myoinositol–labeled mesangial cells is shown in Figure 1. At 15 sec $PGF_{2\alpha}$ enhanced formation of all inositol phosphates (Fig. 1B), compared with cells exposed to vehicle alone (Fig. 1A). Figure 2 shows the magnitude of increase in 1,4-IP_2 and in 1,4,5-IP_3 generation in response to $PGF_{2\alpha}$. The increment in 1,4-IP_2 generation significantly exceeded that of 1,4,5-IP_3.

Following a 15-min exposure to 1 μM $PGF_{2\alpha}$, DAG mass levels (Fig. 3A) were significantly increased (2.33 ± 0.30 pmol/nmol phosphorus) compared with vehicle-treated controls (1.11 ± 0.10 pmol/nmol phosphorus). The change in protein kinase C activity in response to $PGF_{2\alpha}$ is shown in Figure 3B and is expressed as percentage in enzyme activity in cytosolic and membrane fractions compared with vehicle treated controls. Significant decrements in cytosolic PKC activity with concomitant increments in membrane PKC activity (translocation) occurred in response to $PGF_{2\alpha}$.

Time Course of $PGF_{2\alpha}$-Induced PLC Activation. Figure 4 demonstrates the time course of the effect of 1 μM $PGF_{2\alpha}$ on changes in mesangial cell 1,4,5-IP_3 (Fig. 4, upper panel) and DAG (Fig. 4, lower panel) levels. Different patterns of response were obtained. 1,4,5-IP_3 increased and peaked within 15 sec following stimulation with $PGF_{2\alpha}$. At longer incubation times (1, 10, and 15 min) IP_3 levels were no different than controls. In contrast to 1,4,5-IP_3, changes in DAG mass levels occurred in a biphasic pattern. There was an early rise, which coincided with that of 1,4,5-IP_3 at 15–30 sec followed by a drop at 1 min to levels no different than those in parallel unstimulated controls. This was followed by a second progressive rise at 2 and 5 min, which plateaued at 15 min. These differences in the pattern of temporal changes in IP_3 and DAG indicate that there is stimulation of different phospholipases: namely, a PI-specific phospholipase C (PI-PLC) which is stimulated early and accounts for the generation of IP_3 and the early DAG increment (15–30 sec), and a different phospholipase which accounts for the late increments in DAG mass at 2, 5, and 15 min but has no effect on IP_3 generation.

Regulation of $PGF_{2\alpha}$-Induced Formation of Inositol Polyphosphates and DAG. Figure 5 demonstrates the effect of the protein kinase C inhibitor, H7, and the protein tyrosine kinase inhibitor, genistein, on $PGF_{2\alpha}$-induced 1,4,5-IP_3 generation at 15 sec. Preincubation of mesangial cells with H-7 significantly enhanced the $PGF_{2\alpha}$-induced increments in 1,4,5-IP_3. In contrast, preincubation with the protein tyrosine kinase inhibitor genistein had no effect on 1,4,5-IP_3 generation in response to $PGF_{2\alpha}$.

In Figure 6, the effect of Calphostin C, a specific PKC inhibitor that is structurally dissimilar to H-7, on basal 1,4,5-IP_3 generation is shown. Calphostin C dose-dependently enhanced 1,4,5-IP_3 generation. This

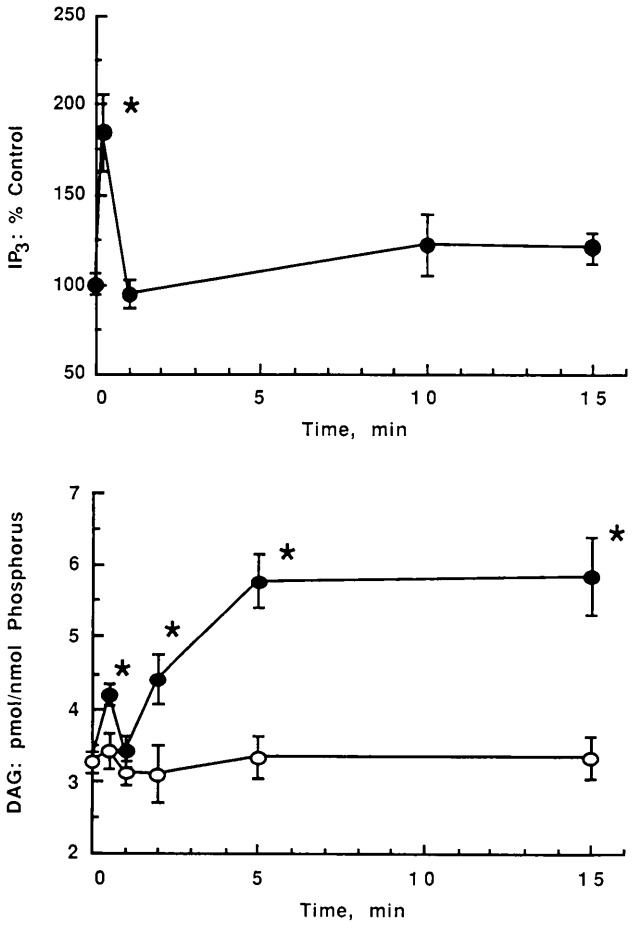

Figure 4. Time course of the effect of 1 μM $PGF_{2\alpha}$ on changes in mesangial cell 1,4-IP_3 (upper panel) and DAG levels (lower panel). 1,4,5-IP_3 (upper panel) peaked early (15 sec) and returned to levels no different than controls at 1, 10, and 15 min. Changes in DAG mass levels (lower panel) occurred in a biphasic pattern. There was an early rise corresponding with the IP_3 peak at 15–30 sec followed by a drop to control levels at 1 min. There was a second progressive rise starting at 2 min, which plateaued by 15 min. For each time point data is expressed as mean ± SEM for n = 3–5 observations. Solid circles, 1 μM $PGF_{2\alpha}$; open circles, vehicle-treated controls. $^*P < 0.05$ compared with simultaneous controls.

effect became significant at 0.1 μM, peaked at 0.3 μM, and plateaued at 0.3–0.5 μM.

Figure 7 demonstrates the effect of H-7, Calphostin C, and genistein on generation of DAG measured 15 min following $PGF_{2\alpha}$ stimulation. Preincubation of mesangial cells with either H-7 or calphostin attenuated the increase in DAG levels in response to $PGF_{2\alpha}$. Preincubation with genistein had no effect.

Figure 8 demonstrates the effect of U-73122 and of pertusis toxin (PTx) on $PGF_{2\alpha}$-induced increments in 1,4,5-IP_3 generation. U-73122 is an inhibitor of receptor-coupled PI-PLC activation through an effect on G_{PLC} (17). Pertussis toxin ribosylates ADP thereby stabilizing the inactive [Gα – GDP]G$\beta\gamma$ complex effectively uncoupling it from the receptor (19). U-73122 alone significantly increased 1,3,4-IP_3 generation above basal levels. In U-73122 pretreated cells production of 1,3,4-IP_3 in response to $PGF_{2\alpha}$ was augmented to levels significantly higher than those obtained in response to $PGF_{2\alpha}$ alone (Fig. 8). In cells pretreated with PTx, production of 1,3,4-IP_3 in response to $PGF_{2\alpha}$ was no different than that observed in cells stimulated with $PGF_{2\alpha}$ alone (Fig. 8).

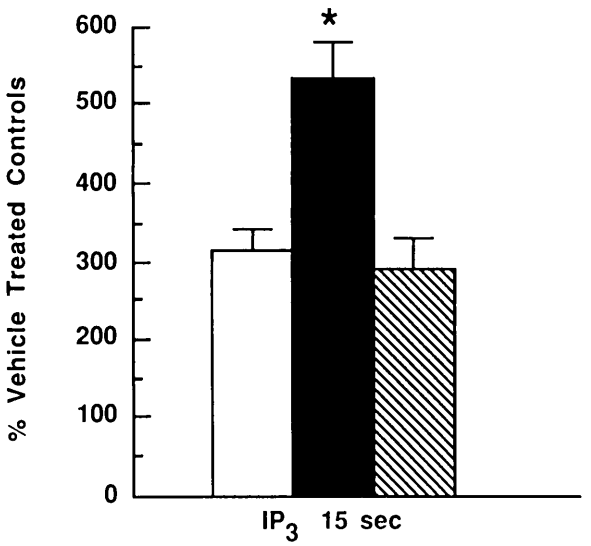

Figure 5. Effect of protein kinase C inhibition using H-7 or of tyrosine kinase inhibition using genistein on $PGF_{2\alpha}$-induced 1,4,5-IP_3 generation. Preincubation of cells with genistein (striped bar) had no effect on $PGF_{2\alpha}$-induced 1,4,5-IP_3 synthesis. Preincubation with the protein kinase C inhibitor H-7 significantly increased $PGF_{2\alpha}$-induced 1,4,5-IP_3 generation. Open bar, $PGF_{2\alpha}$; solid bar, H-7 + $PGF_{2\alpha}$; striped bar, genistein + $PGF_{2\alpha}$. Each bar represents the mean ± SEM for n = 3–14 observations. $^*P < 0.05$ compared with $PGF_{2\alpha}$.

Discussion

Although definitive evidence for the presence of $PGF_{2\alpha}$ receptors in cells of the renal glomerulus (epithelial, endothelial, mesangial) is lacking, their pres-

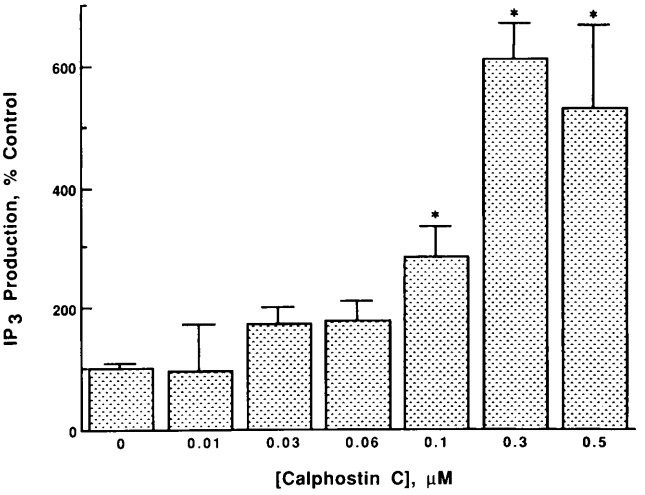

Figure 6. Effect of the specific PKC inhibitor, Calphostin C, on basal 1,4,5-IP_3 generation by mesangial cells. Calphostin C dose-dependently increased IP_3 generation.

ence can be inferred from responses elicited in these cells by $PGF_{2\alpha}$. These responses have best been characterized in glomerular mesangial cells which contract and proliferate in response to $PGF_{2\alpha}$ (3). In addition, $PGF_{2\alpha}$ induces signaling events indicative of PLC activation (i.e., generation of inositol polyphosphates and increase in free cytosolic calcium [8]). The present studies extend these observations and explore: (i) the relative abundance of IP_2 and IP_3 generated in response to $PGF_{2\alpha}$ stimulation, (ii) DAG synthesis and PKC activation, and (iii) mechanisms that regulate PLC activation in response to $PGF_{2\alpha}$.

Our observations indicate that $PGF_{2\alpha}$, increases 1,4-IP_2, 1,4,5-IP_3, and 1,3,4-IP_3 (Fig. 1). Generation of 1,4-IP_2 exceeded that of 1,4,5-IP_3 (Fig. 2). This can be due to hydrolysis by PI-PLC of the PIP substrate. Observations in other systems suggest that hydrolysis of PIP_2 but not of PIP or PI by PLC occurs at physiological intracellular calcium concentration, and that the 1,4,5-IP_3–mediated rise in intracellular calcium promotes PLC to also act on the PIP and PI substrates (20).

$PGF_{2\alpha}$ enhanced mass levels of DAG (Fig. 3A), the endogenous activator of the regulatory domain of PKC, and induced subcellular distribution (increased membrane association) of this enzyme (Fig. 3B). We chose this approach of assessing PKC activation over other methods, such as detection of phosphorylation of endogenous proteins that are believed to serve as specific substrates for PKC (i.e., the 80- and 41-kDa substrates), as it provides a measure of the stability of the PKC-membrane complex, which in turn allows inferences to be made as to how strong a PKC-membrane link develops in response to an agonist. Rat mesangial cells express PKCα, δ, ϵ, and ζ isoforms (21). There is a Ca^{+2}/DAG–sensitive isoform, three Ca^{+2}-independent isoforms, and one isoform insensitive to both Ca^{+2} and DAG. With the possible exception of the PKC ζ isoform, activity of these isoforms is

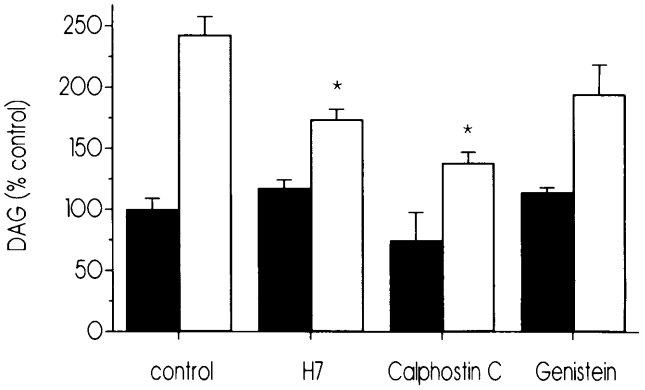

Figure 7. Effect of two structurally dissimilar PKC inhibitors, H-7 and Calphostin C, and of the tyrosine kinase inhibitor, genistein, on DAG generation in response to $PGF_{2\alpha}$. Both H-7 and Calphostin C significantly attenuated DAG generation. Genistein had no effect.

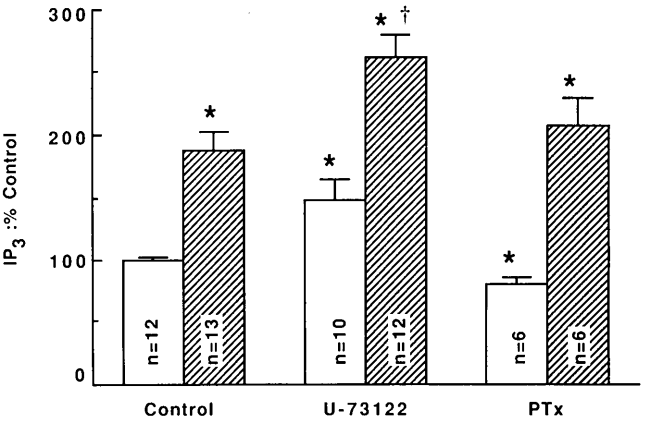

Figure 8. Effect of U-73122, an inhibitor of receptor-coupled PI-PLC activation, and of pertussis toxin (PTx) on $PGF_{2\alpha}$-induced increments in 1,4,5-IP_3 generation. U-73122 significantly increased basal IP_3 production and augmented $PGF_{2\alpha}$-induced IP_3 generation. PTx had no effect on IP_3 generation in response to $PGF_{2\alpha}$. Open bar, inhibitor alone, striped bar, inhibitor + $PGF_{2\alpha}$. Each bar represents the mean ± SEM of n = 6–12 observations. *$P \leq 0.05$ compared with no $PGF_{2\alpha}$ and no inhibitor. †$p < 0.05$ compared with $PGF_{2\alpha}$ alone.

stimulated by phosphatidylserine (PS). The enzyme translocation method employed in the present studies assesses, therefore, activation of all PS-sensitive PKC isoforms.

The time course studies (Fig. 4) indicate that, following the initial $PGF_{2\alpha}$ activation of a PI-PLC, demonstrated by the simultaneous increments in IP_3 and DAG at 15–30 sec, activation of a second phospholipase occurs, as shown by the increments in DAG, but not IP_3 levels at 2, 5, and 15 min. The regulation of the activity of the phospholipase that accounts for the late generation of DAG is apparently different from that of PI-PLC. Thus, PKC inhibition using H-7 augmented phosphorylation of PI-PLC by $PGF_{2\alpha}$, assessed as generation of IP_3 at 15 sec (Fig. 5). Moreover, the specific PKC inhibitor, Calphostin C, augmented basal IP_3 generation (Fig. 6). In contrast, both H-7 and Calphostin C attenuated generation of DAG in response to $PGF_{2\alpha}$ (Fig. 7). This indicates that the late DAG generation is due to a PKC-dependent phospholipase. Likely candidates are a phosphatidyl choline–specific phospholipase C or the concerted action of a phospholipase D and a phosphatidate phosphohydrolase. As activation of PLD is PKC dependent (22) and $PGF_{2\alpha}$ can activate PLD (23), this phospholipase is likely to account for the late sustained generation of DAG. Thus, PLD activation may be a secondary response to PIP_2 hydrolysis and DAG-dependent PKC activation induced by $PGF_{2\alpha}$.

The stimulatory effect of PKC inhibition on IP_3 generation (Fig. 5 and 6) indicates that PKC exerts an inhibitory effect on PI-PLC. This is in agreement with observations made by Mène *et al.*, who demonstrated that activation of PKC by phorbol esters abrogates the effect of $PGF_{2\alpha}$ on IP_3 formation in cultured mesangial cells (8). Activation of cyclic AMP-dependent protein

kinases may also inhibit receptor-coupled activation of PI-PLC (24), but this is an unlikely mechanism with respect to $PGF_{2\alpha}$, as this eicosanoid lowers cAMP levels (25).

Activation of inositol phospholipid–specific PLC can occur as a result of an interaction with transmembrane receptors that possess ligand-activated cytoplasmic tyrosine kinase activity, as has been demonstrated for the PI-PLC-γ_1 isoform in response to peptide growth factors (i.e., EGF and PDGF) (26). In addition to tyrosine phosphorylation–dependent activation, a variety of agonists have been shown to regulate PI-PLC activity through guanine nucleotide binding (G) proteins. Both pertussis toxin-sensitive and insensitive G proteins have been shown to regulate PI-PLC activation. Of these, pertussis toxin–sensitive G proteins specifically activate the β_1 isoenzyme but have no effect of the γ_1 and δ_1 isoenzymes (26). Our observations demonstrate that in mesangial cells $PGF_{2\alpha}$-induced PI-PLC activation, assessed as changes in IP_3 generation, is independent of protein tyrosine phosphorylation, as the protein tyrosine kinase inhibitor, genistein, had no effect on IP_3 generation in cells pretreated with this inhibitor (Fig. 5). This raises the possibility that a G protein mediates activation of PI-PLC in response to $PGF_{2\alpha}$, as has been proposed by Smith *et al.*, who suggested that $PGF_{2\alpha}$ receptors are associated with a specific guanine nucleotide regulatory protein, linked to activation of PLC (27). The recent observation that the $PGF_{2\alpha}$ receptor consists of a heptahelical transmembranous domain further supports this suggestion (28). To explore this, we studied the effect of pertussis toxin and of the aminosteroid compound U-73122, which inhibits receptor-coupled PI-PLC activation through an apparent effect on G_{PLC} (the GTP-binding protein that regulates phospholipase C activity) (17). Our observations indicate that these compounds had different effects on PI-PLC activation by $PGF_{2\alpha}$: PTx had no effect, while U-73122 augmented the $PGF_{2\alpha}$-induced PI-PLC activation (Fig. 8). The inability of PTx to inhibit the $PGF_{2\alpha}$-induced IP_3 response points to a mechanism of PI-PLC activation that is dependent on a pertussis toxin–insensitive G protein. A likely candidate is the pertussis toxin insensitive G protein, designated G_q. Indeed, recent studies using NIH-3T3 fibroblasts have shown that $PGF_{2\alpha}$ activates MAP kinase *via* a G_q protein coupled pathway (29). The inability of U-73122 to attenuate or block PI-PLC activation by $PGF_{2\alpha}$ is puzzling. Although concentrations known to inhibit receptor-coupled PI-PLC activation were employed (20–50 μM for 18 hr) (17), this compound significantly increased basal 1,4,5-IP_3 production and augmented its production in response to $PGF_{2\alpha}$ (Fig. 8). This indicates that U-73122 promoted PI-PLC activity. Therefore, its use in our system did not elucidate the role of G proteins in regulating PI-PLC activation by $PGF_{2\alpha}$.

Whether $PGF_{2\alpha}$ can act *via* the mesangial cell TxA_2 receptor is unknown. Recent studies on characterization of mouse PGF receptor reported that this receptor consists of 366 amino acid residues with seven putative transmembrane domains. The sequence homology analysis revealed that it shares significant sequence similarly with other members of G protein–coupled receptors especially with the mouse TxA_2 receptor (28). The sequence identity between TxA_2 receptor and PGF receptor was 47.8%. RNA blot and *in situ* hybridization analyses demonstrated abundant expression of the PGF receptor in the kidney (28). On the basis of these observations, it can be argued that in forms of glomerular injury resulting in high intraglomerular levels of $PGF_{2\alpha}$ and TxA_2, an additive effect of these eicosanoids may occur by virtue of receptor homologies and potentiate mesangial cell responses.

In summary, our studies indicate that $PGF_{2\alpha}$ activates the intracellular signaling enzymes PLC and PKC. IP_2 generation in response to PLC activation exceeds that of IP_3 generation and likely occurs due to an effect of PI-PLC on the PIP substrate. $PGF_{2\alpha}$ activates at least two PLC species: a PI-PLC which is under negative feed back regulatory control by PKC and a different phospholipase which is under positive regulatory control by PKC and accounts for sustained generation of DAG. $PGF_{2\alpha}$-induced PI-PLC activation is independent of protein tyrosine phosphorylation and is mediated by pertussis toxin–insensitive G proteins.

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