

Differential Regulation of Renal Phospholipase C Isoforms by Catecholamines

Pei-Ying Yu, Laureano D. Asico, Gilbert M. Eisner, and Pedro A. Jose

Departments of Pediatrics and Physiology and Biophysics, Georgetown University School of Medicine, Washington, DC 20007

Abstract

Dopamine and D_1 agonists and NE all increase phosphatidyl inositol-specific phospholipase C (PLC) activity, but whereas dopamine produces a natriuresis, NE has an antinatriuretic effect. To determine if catecholamines differentially regulate the expression of PLC isoforms, we infused fenoldopam, a D_1 agonist, or pramipexole, a D_1/D_2 agonist, intravenously or infused fenoldopam or NE into the renal artery of anesthetized rats. After 3–4 h of infusion, when the expected natriuresis (fenoldopam or pramipexole) or antinatriuresis (NE) occurred, the kidneys were removed for analysis of PLC isoform protein expression and activity. Western blot analysis revealed that in renal cortical membranes, fenoldopam and pramipexole increased expression of $PLC\beta_1$ and decreased expression of $PLC\gamma_1$; $PLC\delta$ was unchanged. In the cytosol, pramipexole and fenoldopam increased expression of both $PLC\beta_1$ and $PLC\gamma_1$. No effects were noted in the medulla. A preferential D_1 antagonist, SKF 83742, which by itself had no effect, blocked the effects of pramipexole, thus confirming the involvement of the D_1 receptor. In contrast, NE also increased $PLC\beta_1$ but did not affect $PLC\gamma_1$ protein expression in membranes. The changes in PLC isoform expression were accompanied by similar changes in PLC isoform activity. These studies demonstrate for the first time differential regulation of PLC isoforms by catecholamines. (*J. Clin. Invest.* 1995; 95:304–308.) Key words: dopamine agonist • dopamine receptor • norepinephrine • phospholipase C • kidney

Introduction

Dopamine can inhibit transport by cAMP-dependent and -independent mechanisms (1–5). We and others have reported that dopamine and D_1 agonists can inhibit Na^+/H^+ exchange activity by a guanine nucleotide binding protein (G-protein)¹-dependent/cAMP-independent mechanism; in these experiments, Na^+/H^+ exchange activity was inhibited even when formation of cAMP/protein kinase A was prevented (1, 2). Another

cAMP-independent pathway of sodium transport inhibition may be via a putative dopamine receptor, which thus far has not been cloned (6, 7). This putative receptor shares some properties with the D_{1A} and D_{1B} receptors, since all are blocked by D_1 antagonists (e.g., SCH 23390 and SKF 83742) and stimulated by D_1 agonists (e.g., fenoldopam). This receptor, unlike the cloned D_{1A} and D_{1B} receptors that are linked to stimulation of adenylyl cyclase, is linked to the stimulation of phosphatidyl inositol-specific phospholipase C (PLC) and not to adenylyl cyclase (6–11). Dopamine has also been demonstrated to inhibit Na^+/K^+ ATPase activity by stimulating PLC activity (4, 5). However, angiotensin II and α -adrenergic agonists, which also stimulate PLC activity, increase Na^+/K^+ ATPase and Na^+/H^+ exchange activity (2, 12–17).

PLC isoforms have been linked to specific receptors, G-proteins, and tyrosine kinases (18, 19). For example, stimulation of G-protein linked cell surface receptors such as α -adrenergic and serotonin receptors has been linked to increased $PLC\beta_1$ without changes in $PLC\gamma_1$ activity (20–23). The PLC isoform linked to angiotensin II is not clear but it is not $PLC\gamma_1$ (24). However, the linkage of these neurotransmitters to PLC isoforms was studied in reconstituted systems and not in their innate environment. The linkage of D_1 dopamine receptors to PLC isoforms has not been reported. We therefore examined in the kidney the linkage of D_1 dopamine receptors to PLC isoforms and compared it with NE to determine if differential regulation of PLC isoforms by catecholamines occurs.

Methods

Rats were maintained on standard rat chow until the day of experiment. The rats were anesthetized with pentobarbital (50 mg/kg body wt, i.p.), placed on a heated table to maintain rectal temperature between 36 and 37°C, and tracheotomized (PE-240). Anesthesia was maintained by the infusion of pentobarbital at 0.8 mg/100 g body wt per h. Catheters (PE-50) were placed into the external jugular and femoral veins and left femoral artery. Systemic arterial pressure was monitored electronically using Cardiomax II (Columbus Instruments, Columbus, OH). Suprapubic cystostomy was performed for urine collection. Fluid losses during surgery were replaced with 5% albumin at 1% body weight over 30 min. The jugular vein was cannulated (PE-50) for the infusion of normal saline solution at a rate of 5 ml/100 g body wt for 30 min, followed by a rate of 1.8 ml/100 g body wt per h until the end of the experiment. After an equilibration period of 120 min, the first 60-min urine collection for clearance determinations was begun and served as the baseline period. The first group of rats was divided into those receiving normal saline (1.2 ml/h i.v.), the D_1 agonist fenoldopam (5 μ g/kg per minute at 1.2 ml/h i.v.), and the D_1/D_2 agonist pramipexole (5 μ g/kg per min at 1.2 ml/h i.v.). The second group of rats was divided into those receiving normal saline (1.2 ml/h i.v.), the D_1/D_2 agonist pramipexole (5 μ g/kg per min at 1.2 ml/h i.v.), the preferential D_1 antagonist SKF 83742 (5 μ g/kg per min at 1.2 ml/h i.v.), or a combination of pramipexole and SKF 83742 (each at 5 μ g/kg per min at 1.2 ml/h i.v.). In both of the above groups of rats, the vehicle- and drug-treated animals were always studied at the same time.

In a third group of rats, the right suprarenal artery was catheterized and normal saline was infused at 0.2 ml/h. After the first 60-min collection period for clearance determinations, the infusate was changed to

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Address correspondence to Pei-Ying Yu, M.D., Instructor in Pediatrics, Georgetown University Children's Medical Center, 3800 Reservoir Rd., NW, Washington, DC 20007. Phone: 202-687-8675; FAX: 202-687-7161.

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1. Abbreviations used in this paper: G-protein, guanine binding protein; PIP_2 , phosphatidyl inositol-4,5-bisphosphate; PLC, phospholipase C.

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fenoldopam (1 $\mu\text{g/kg}$ per min) or NE (1 $\mu\text{g/kg}$ per min) at 0.2 ml/h. In this third group of rats, urine was collected from the right and left ureters. After 3–4 h, during which time the natriuresis (dopamine agonist) or antinatriuresis (NE) was expected to be evident (and verified subsequently by actual measurements of sodium excretion), the kidneys were removed for analyses. The uninfused left kidney served as a concurrent control for the right infused kidney.

Preparation of and purification of cytosol and membranes from kidney. The kidney cortex was separated from the medulla and homogenized (1 g/2 ml) in (mM) 50 Tris-Cl, pH 7.4, containing 1 EDTA, 5 PMSF, 0.1 diisopropyl fluorophosphate, 0.1 DTT, 100 NaCl, and 250 sucrose (buffer A) using a polytron homogenizer. The homogenate was centrifuged at 100,000 g for 30 min at 4°C. The supernatant represented the cytosol fractions. The particulate fractions were then washed three times, first with 50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 2 M KCl, second with 20 mM Tris-HCl, 1 mM EDTA, and third with buffer A. The protease inhibitors trypsin inhibitor, leupeptin, and aprotinin (10 $\mu\text{g/ml}$) were added to all the buffers. The final pellets were then resuspended in 4 ml of 50 mM Tris-HCl, pH 7.0, containing 1% sodium cholate and stirred overnight on ice. After centrifugation at 100,000 g for 30 min, the supernatant was obtained and designated as crude extract of membrane PLC. The PLC in the cytosol and membrane fractions were partially purified according to the methods of Ryu et al. (25) and Carter et al. (26). In brief, the pH of cytosol fractions was adjusted to 5.0 by 1 M acetic acid with stirring on ice for 30 min. The precipitates were collected by centrifugation at 15,000 rpm for 30 min and dissolved in the buffer A. The membrane fractions, extracted by 1% sodium cholate, were mixed with DEAE cellulose (2 ml/sample) on ice for 1 h. Thereafter, the mixture was poured onto minicolumns (2 ml). After washing the unbound proteins, the columns were eluted with buffer A containing 110 or 150 mM KCl. PLC β_1 is eluted by 110 mM KCl but not by 150 mM KCl (25). The eluates were concentrated by Amicon (Beverly, MA) concentrators. The protein concentrations of the samples were determined by the Bradford method (27).

Measurement of PLC activity. The 110- and 150-mM KCl eluates and cytosol were assayed for PLC activity according to the method of Nishibe et al. (28). In brief, the assay was performed in 50 μl of reaction mixture containing 40,000 cpm of phosphatidyl inositol-4,5-bisphosphate ($[^3\text{H}]\text{PIP}_2$), 0.15% *n*-octyl-beta-D glucopyranoside, 0.05% Triton X-100, (mM) 0.8 CaCl_2 , 0.8 EGTA, 35 NaH_2PO_4 , pH 6.8, 70 KCl, and 10–40 μg of protein. The reaction mixtures were incubated for 15 min at 37°C and terminated by adding a stop solution containing 100 μl of 1% BSA and 500 μl of 10% trichloroacetic acid. PIP_2 -hydrolyzing radioactivity was determined by liquid scintillation counting. The results of the activity were expressed as cpm/mg protein/min.

Immunoblot analysis. The partially purified cytosol and membranes were mixed with Laemmli sample buffer, boiled for 5 min, and subjected to electrophoresis on 7.5% SDS-PAGE. Rat brain cytosol was used for positive control. The proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes. The transblot sheets were blocked with 1% nonfat dry milk in 10 mM Tris-HCl, pH 7.5, containing saline and 0.05% Tween-20 (TBST buffer), and incubated with diluted monoclonal antibodies to PLC isoforms for 2 h at room temperature or overnight at 4°C. The sheets were washed with TBST buffer three times and then incubated with diluted secondary antibody peroxidase-conjugated affinity-purified goat anti-mouse IgG for 2 h at room temperature. The immunoblots were extensively washed and developed with a solution containing 5 mg of 3,3'-diaminobenzidine and 3.3 μl of 30% hydrogen peroxide in 10 ml of 50 mM Tris-Cl, pH 7.5. In some cases, enhanced chemiluminescence (ECL) Western blot detection reagents (RPN 2106) were used, and the immunoblots were examined after exposed to Kodak film. The immunoblots were quantified using Quantiscan (Biosoft, Ferguson, MO). The percent area of each blot was quantified with the total area arbitrarily set at 100%.

We obtained monoclonal antibodies for the PLC isoforms of proven specificity that have been used and characterized by others (29, 30). In our studies, the absence of cross-reactivity was easily determined since the molecular size of PLC β_1 (150 kD) was different from that of PLC γ_1

(145 kD). Absence of cross-reactivity and specificity were further verified by immunoprecipitation of PLC γ_1 or PLC β_1 . Furthermore, PLC β_1 was not detected in the 150-mM KCl eluate. Normal mouse IgG also did not show any bands at 150 and 145 kD.

Materials. Materials were obtained from the following manufacturers: adult male Wistar Kyoto (WKY) rats (250–400 g, Taconic Farms, Inc., Germantown, NY); D_1 agonist, fenoldopam, and D_1 antagonist, SKF 83742 (SmithKline Beecham Pharmaceuticals, Philadelphia, PA); D_1/D_2 agonist, pramipexole, (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT); PLC isozyme antibodies (Upstate Biotechnology Inc., Lake Placid, NY); goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs, Inc., West Grove, PA); ECL Western blotting detection reagents, RPN2106 (Amersham Corp., Arlington Heights, IL); $[^3\text{H}]\text{PIP}_2$ (Dupont-NEN, Boston, MA). All other chemicals were from Sigma Chemical Company (St. Louis, MO).

Results

Renal function studies. As expected, the intravenous infusion of the D_1/D_2 agonist, pramipexole, increased fractional sodium excretion (0.19 ± 0.11 vs. $0.89 \pm 0.22\%$, $n = 6$) and the right intrarenal arterial infusion of the D_1 agonist, fenoldopam, increased fractional sodium excretion (0.35 ± 0.13 vs. $0.90 \pm 0.27\%$, $n = 6$). These increases were significant compared with basal values or with vehicle-treated rats ($P < 0.05$ ANOVA for repeated measures, Scheffe's test). Mean arterial pressure and creatinine clearance did not change. The D_1 antagonist, SKF 83742, alone ($n = 6$) did not affect mean arterial pressure or creatinine clearance; however, it did block the diuretic and natriuretic effect of pramipexole. These changes are similar to those reported from our laboratory and those of others (31, 32). The right intrarenal arterial infusion of NE infusion, as expected, decreased absolute sodium excretion (2385 ± 794 to 1574 ± 836 , $n = 5$) ($P < 0.05$ ANOVA for repeated measures); fractional sodium excretion was slightly but not significantly decreased (1.28 ± 0.40 vs. $0.90 \pm 0.38\%$), probably due to a slight but insignificant fall in glomerular filtration rate.

Immunoblot analysis of PLCs in renal cortical cytosol and membrane fractions. After administration of the drugs outlined above, kidney cortex or medulla was prepared as described in Methods. The partially purified cytosol and membranes were subjected to 7.5% of SDS-PAGE gels. We found that at least three PLC isozymes (PLC β_1 , PLC γ_1 , and PLC δ_1) were expressed in kidney, and their molecular masses were the same as those reported for the rat brain, 150, 145, and 85 kD, respectively (not shown). The intravenous infusion of pramipexole, a D_1/D_2 agonist, fenoldopam, a D_1 agonist, or vehicle was associated with increased expression of PLC β_1 and PLC γ_1 in renal cortical cytosolic fractions. In contrast, in renal cortical membranes, the expression of PLC β_1 increased but the expression of PLC γ_1 decreased (Fig. 1, A and B); PLC δ was unchanged (data not shown). There were no changes in any of the PLC isoforms tested in renal medullary tissues (data not shown).

We chose to examine further the effect on PLC isoforms of the D_1/D_2 agonist, pramipexole, since dopamine has effects on both the D_1 and D_2 receptors (8). Pramipexole was chosen over dopamine, however, since the latter has actions on nondopamine receptors as well (8, 31). To determine specificity of the pramipexole effect, concurrent studies were performed using the dopamine antagonist SKF 83742. SKF 83742 is a full D_1 antagonist but only a partial D_2 antagonist. In LTK $^-$ cells stably transfected with the rat $\text{D}_{2\text{long}}$ cDNA, concentrations of SKF

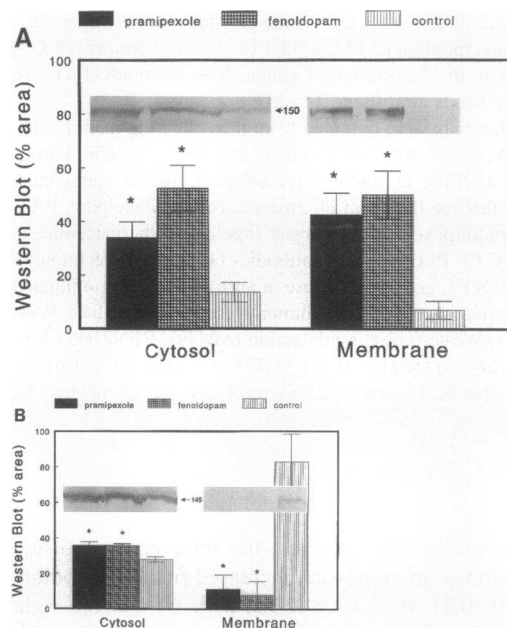


Figure 1. Immunoblot of cytosol and membrane fraction from rat kidney cortex using monoclonal antibodies against PLC β_1 (A) or PLC γ_1 (B) after 3–4 h of intravenous infusion of the D₁/D₂ agonist pramipexole, the D₁ agonist fenoldopam, or vehicle (control). The representative immunoblots are shown at the top. Densitometric analysis of immunoblots shows that compared with vehicle-treated rats (control group), pramipexole and fenoldopam increased PLC β_1 in cytosol and membrane; these ligands also increased PLC γ_1 in cytosol but decreased it in membranes ($n = 4$ –5 per group). Data are means \pm SE. * $P < 0.05$ vs. control, ANOVA and Scheffe's test.

83742 as high as 10^{-4} M were able to displace $< 50\%$ of specific 125 I-spiroperidol binding (data not shown); in LTK⁺ cells stably transfected with the rat D_{1A} cDNA, the same concentrations of SKF 83742 completely displaced specific 125 I-SCH 23982 binding. Pramipexole increased the expression of PLC β_1 whereas it decreased the expression of PLC γ_1 in renal cortical membranes; the expression of both PLC isoforms was increased in cytosolic fraction (Fig. 2, A and B), confirming the studies shown in Fig. 1, A and B. The changes were blocked by the antagonist SKF 83742, which by itself had no effect (Fig. 2, A and B). To minimize variability of the experimental procedures, sets of vehicle- and drug-treated rats were always studied concurrently.

Determination of PLC activity. To determine if the changes in PLC isoform protein expression were associated with changes in enzyme activity, additional studies were performed. To minimize extrarenal influences, fenoldopam was infused into the right renal artery; the left kidney served as control. Western blotting of the 110- and 150-mM KCl eluate confirmed the studies of Ryu et al. (25), showing that 110 mM KCl eluted PLC β_1 and was not detectable in the 150-mM KCl eluate; PLC γ_1 was found in both 110- and 150-mM KCl eluate (Fig. 3, A and B). Compared with the noninfused left kidney, in renal cortical membranes fenoldopam increased PLC activity (29%, Table I) of the 110-mM KCl eluate. This increase in PLC activity induced by fenoldopam represents PLC β activity since PLC β_1 expression increased 178%, whereas PLC γ_1 expression decreased (-53%) in this eluate (Fig. 3). PLC activity in the 150-mM KCl eluate, which represents PLC γ activity (PLC β_1 ,

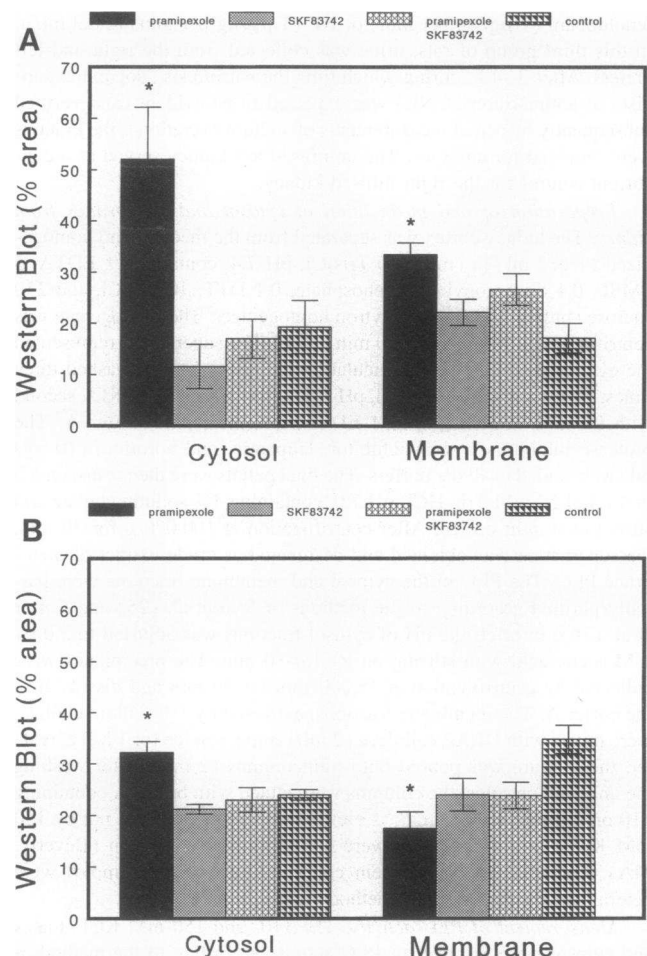


Figure 2. Immunoblot of cytosol and membrane fraction from rat kidney cortex using monoclonal antibodies against PLC β_1 (A) or PLC γ_1 (B) after 3–4 h of intravenous infusion of the D₁/D₂ agonist pramipexole, the full D₁, partial D₂ antagonist SKF 83742, the co-infusion of pramipexole and SKF 83742, or vehicle (control group). Densitometric analysis of immunoblots shows that compared with vehicle-treated rats, pramipexole increased PLC β_1 in cytosol and membrane; this agonist also increased PLC γ_1 in cytosol but decreased it in membranes. The effects were blocked by SKF 83742, which by itself had no effect ($n = 4$ –5 per group). Data are means \pm SE. * $P < 0.05$ vs. control, ANOVA and Scheffe's test.

was not detectable in this fraction), was decreased by fenoldopam (-42%). The increase in PLC β activity associated with fenoldopam in the 110-mM KCl eluate is an underestimate since PLC γ activity, which decreased, contributed 40% of PLC protein in this fraction. In agreement with previous reports using reconstituted cell systems (reviewed in reference 22), in renal tubular membranes, NE also increased PLC β expression (not shown) and activity but had no effect on PLC γ activity (Table II). PLC activity in cytosol was increased by both fenoldopam and NE.

Discussion

In renal cortical homogenates, dopamine and D₁ agonists (D₁ receptors in the kidney are most abundant in proximal tubules) can stimulate phosphoinositide hydrolysis by activation of phosphatidylinositol-specific PLC (6, 7). The ability of dopamine

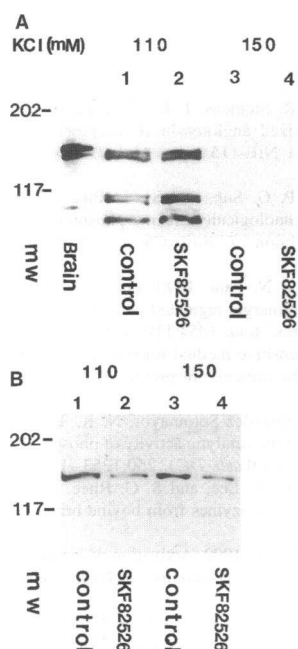


Figure 3. Representative immunoblot of the 110- or 150-mM KCl elution of partially purified PLC from rat renal cortical membranes using monoclonal antibodies against PLC β_1 (A) or PLC γ_1 (B) after 3–4 h of right intrarenal infusion of the D $_1$ agonist SKF 82526 (fenoldopam). Lanes 1 and 2 show the immunoblots of the 110-mM KCl eluate; lanes 3 and 4 show the immunoblots of the 150-mM KCl eluate. The 110-mM KCl eluate (lanes 1 and 2) contained PLC β_1 (the band at 150 kD; the bands with < 117 probably represents degradation products). The 150-mM KCl eluate (lanes 3 and 4) contained exclusively PLC γ_1 (compare A and B, lanes 3 and 4). In five studies, SKF 82526 increased PLC β_1 (the % area in the control was 36 compared with 64 after SKF 82526) but decreased PLC γ_1 expression (the % area in the control was 68 compared with 32 after SKF 82526).

to initiate phosphoinositide hydrolysis has been confirmed in the kidney (2, 4, 33), brain striatum (11, 34), and the retina (10). Furthermore, cholera and pertussis toxin-insensitive G-proteins were involved (7).

Several PLC enzymes have been purified, molecularly cloned, and sequenced (18, 19). These isoforms of PLC have been classified into three families, PLC β , PLC γ , and PLC δ with several members in each (e.g., PLC β_1 , PLC β_2 , PLC β_3). PLC has been linked to pertussis sensitive and insensitive G-proteins and tyrosine kinases (18, 19, 23, 35). The G $_q$ family of G-proteins, which are pertussis toxin insensitive, have been linked to activation of PLC β_1 whereas tyrosine kinases activate PLC γ_1 (36, 37). The receptors that activate PLC via G $_q$ include thromboxane A $_2$, bradykinin, angiotensin II, histamine, vasopressin, acetylcholine muscarinic (M $_1$, M $_3$, and M $_5$), α_1 -adrenergic (α_{1B} and α_{1C}), α_2 -adrenergic (α_{2A}), and serotonergic (5-HT $_{1C}$) receptors (19, 20, 22, 28, 38–42). PLC β isoforms (PLC β_3 > PLC β_2 > PLC β_1) can also be activated by G-protein β/γ -subunits independently of α -subunits (43). However, these studies were performed in reconstituted systems. The link-

Table I. Effect of the D $_1$ Agonist Fenoldopam on Activity of PLC in Rat Kidney Cortex Cytosol and Membrane

Protein fractions	Experimental condition		% of control
	Fenoldopam	Control	
	cpm/mg protein/min		
Cytosol	4,905 \pm 1,006	3,711 \pm 729	30.82 \pm 3.85*
Membrane			
110 mM KCl	38,785 \pm 6,247	31,019 \pm 6,182	28.60 \pm 6.86**
150 mM KCl	17,675 \pm 1,547	30,890 \pm 2,395	–41.88 \pm 6.48*

The PIP $_2$ -hydrolyzing activity of PLC was measured with [3 H]PIP $_2$ as the substrate. Results are the mean \pm SE of five experiments. * P < 0.01 compared with control, paired t test.

Table II. Effect of NE on Activity of PLC in Rat Kidney Cortex Cytosol and Membrane

Protein fractions	Experimental condition		% of control
	NE	Control	
	cpm/mg protein/min		
Cytosol	2,408 \pm 266	2,184 \pm 229	10.20 \pm 2.10*
Membrane			
110 mM KCl	41,166 \pm 4,556	31,515 \pm 2,147	29.40 \pm 6.78*
150 mM KCl	20,637 \pm 3,435	20,814 \pm 3,781	–0.24 \pm 1.7

The PIP $_3$ -hydrolyzing activity of PLC was measured with [3 H]PIP $_2$ as the substrate. Results are the mean \pm SE of five experiments. * P < 0.05 compared with control, paired t test.

age of these ligands to PLC isoforms has not been examined in intact cells. Our studies show for the first time that a G-protein linked receptor stimulates PLC β_1 expression and activity in its natural environment. Thus the stimulation of D $_1$ receptors increases PLC β_1 expression and activity in renal cortical tubules like the other G $_q$ -protein linked receptors (19, 22). Furthermore, our studies show for the first time that a G-protein linked receptor can also affect the PLC γ_1 isoform expression and activity. Of greater interest is the finding that two chemically unrelated dopamine receptor agonists, the D $_1$ receptor agonist fenoldopam and the D $_1$ /D $_2$ receptor agonist pramipexole, decreased PLC γ_1 expression and activity in the membrane at a time when PLC γ expression and activity were increased in the cytosol. In contrast, NE, although increasing PLC β_1 expression and activity, did not affect PLC γ activity in membrane. PLC activity in cytosol was increased similar to that noted with fenoldopam. α_1 -Adrenergic receptors have been reported not to affect PLC γ_1 expression in reconstituted cell systems; however, a distinction between membrane and cytosol fraction was not made (20).

Besides linkage of D $_1$ -like receptors to PLC via pertussis-insensitive G-proteins, one of the D $_1$ receptors linked to stimulation of adenylyl cyclase (D $_{1A}$) also stimulated phosphoinositol hydrolysis via cholera toxin-sensitive G-proteins in murine fibroblasts transfected with the human or rat D $_{1A}$ cDNA (44). Whether the increased expression of PLC β_1 in the kidney due to occupation of D $_1$ -like receptors occurs via the putative D $_{1PLC}$ or the D $_{1A}$ receptor could not be determined in these studies. However, PLC β_1 is not linked to cholera toxin-sensitive G-proteins (reviewed in reference 22).

In summary, we have demonstrated that two D $_1$ agonists increased expression of PLC β_1 but decreased expression of PLC γ_1 in renal cortical membranes. NE also increased PLC β expression and activity but had no effect on PLC γ activity in renal cortical membranes. The changes in protein expression were accompanied by similar directional changes in PLC isoform activity. No changes in PLC isoforms were noted in the renal medulla. PLC δ expression was not affected by D $_1$ agonists in either cortex or medulla. The functional significance of this differential regulation of PLC isoforms remains to be determined. It remains to be proved whether differential activity of PLC isoforms can explain the differential effects of dopamine and NE on sodium transport.

Note Added in Proof. While this paper was in preparation, Marrero et al. reported that angiotensin II stimulates phosphorylation of PLC γ_1 (Marrero, M. B., W. G. Paxton, J. L. Duff,

B. C. Berk, and K. E. Bernstein. 1994. Angiotensin II stimulates tyrosine phosphorylation of phospholipase C- γ 1 in vascular smooth muscle cells. *J. Biol. Chem.* 269:10935–10939).

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