Abstract—This study was designed to delineate the involvement of phospholipase C (PLC) and phospholipase D (PLD) in transmural pressure control of renin synthesis and secretion. Primary cultures of rat juxtaglomerular (JG) cells were applied to a transmural pressure-loading apparatus for 12 hours, and the renin secretion rate (RSR), active renin content (ARC), and total (active + inactive) renin content (TRC) were determined. Under control conditions (n=5), transmural pressure decreased RSR (78.1±3.0 and 64.6±4.4% for 0 or 40 mm Hg, respectively; P<0.05) and ARC (42.8±3.3 and 26.0±3.9 ng of angiotensin I per hour per million cells for 0 or 40 mm Hg, respectively). Treatment with PLC inhibitors, 2-nitro-4-carboxyphenyl-N,N-diphenyl-carbamate (200 μmol/L) and U73122 (10 μmol/L) did not alter RSR but prevented the RSR decrease with transmural pressure, whereas neither 2-nitro-4-carboxyphenyl-N,N-diphenyl-carbamate nor U73122 altered ARC, TRC, or the decrease in ARC with transmural pressure. Experiments were also performed using JG cells (n=5) treated with a PLD inhibitor, 4-(2-aminoethyl)-benzensulfonyl fluoride (AEBSF, 100 μmol/L). Treatment with AEBSF did not influence basal levels of RSR, ARC, and TRC or the RSR decrease with transmural pressure. However, AEBSF did inhibit the decrease in ARC with transmural pressure. These results indicate that transmural pressure inhibits renin secretion via PLC-dependent pathways and prevents conversion of inactive renin to active renin via PLD-dependent mechanisms in JG cells. (Hypertension. 2002;39[part 2]:363-367.)

Key Words: renin-angiotensin system ■ phospholipase

Acute elevation of arterial pressure elicits a prompt inhibition of renin secretion from juxtaglomerular (JG) cells. Using primary cultured JG cells, we recently demonstrated that a chronic transmural pressure load not only suppresses renin secretion from JG cells but also inhibits prorenin processing in JG cells.1 In addition, chronic administration of thapsigargin and caffeine, which deplete intracellular calcium stores, prevented the inhibition of renin secretion and prorenin processing in JG cells during sustained high pressure.1 These data suggest that an intracellular calcium store-dependent mechanism plays an important role in pressure control of renin regulation during sustained high pressure.

Phospholipase C (PLC) and phospholipase D (PLD) are involved in the second messenger pathways in cellular responses to angiotensin II (Ang II)2–3 and mechanical stimulation,4–7 and activation of these enzymes leads to the generation of inositol 1,4,5-trisphosphate (IP3), which promotes calcium release from intracellular calcium stores.8 Recent studies have demonstrated that PLC contributes to increases in inositol phosphates evoked by mechanical distention in renin-expressing As4.1 cells9 and pressure-mediated vasoconstriction in afferent arterioles,10 which are metaplastically transformed to JG cells.11,12 However, there is no experimental evidence indicating that PLC and PLD play roles in modulating pressure control of renin synthesis and secretion in JG cells.

The purpose of this study was to test the hypothesis that transmural pressure controls renin synthesis and secretion through activation of PLC and PLD in JG cells. In the presence and absence of enzyme inhibitors selective for PLC or PLD, JG cells were subjected to a transmural pressure-loading apparatus devised in our laboratory.1

Methods

Primary Culture of Rat JG Cells

In accordance with the guidelines and practices established by the Keio University Animal Care and Use Committee, JG cells were isolated from kidneys of male Sprague-Dawley rats (100 to 150 g) as previously described.1,13,14 JG cells were suspended at 106 cells/mL in culture medium consisting of RPMI 1640 with 25 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid, 0.3 g/L L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, 0.66 U/L insulin, and 10% fetal bovine serum. Cell number was determined using a Coulter counter (Miami, FL). The suspended cells were distributed in 1-mL aliquots into individual wells of 8-well chamber slides containing 1 mL of culture medium and incubated at
37°C, JG cells had a 48-hour rest period before the beginning of the experiments. Immunofluorescence staining for renin confirmed 87±4% of the cells (n=5 primary cultures) to be positive for renin at 60 hours after isolation.1,13,14

Pressure-Loading JG Cells
Pressure was loaded on JG cells with a minimal contribution from shear stress or stretch, as reported previously. The 8-well chamber slides were placed in a sanitary pressure vessel (model DV-5-ST; Advantec Toyo) prewarmed to 37°C. The pressure vessel was then sealed tightly and connected to tubing attached to a 3-way rotary valve, a sphygmomanometer, and a pressure valve. Compressed helium was pumped in to raise the internal pressure. The sanitary pressure vessel was then placed in the incubator, and the internal temperature was kept constant at 37°C. During the experiments, the loaded pressure level was monitored with a sphygmomanometer. The partial pressure of oxygen and the pH of the medium averaged 155±4 mm Hg and 7.4±0.1, respectively, and were kept constant throughout the experiments. In addition to atmospheric pressure, JG cells were subjected to transmural pressures of 0 and 40 mm Hg in the present study.

Measurement of Renin Secretion Rate, Active Renin Content, and Total Renin Content in JG Cells
The renin secretion rate (RSR), active renin content (ARC), and total (active + inactive) renin content (TRC) were measured in JG cells as described previously.1 In brief, after the culture medium had been removed, the cells were washed twice with prewarmed phosphate-buffered saline (PBS). Each well was filled with 1 mL of Ca2+-containing PBS and placed in the pressure-loading apparatus. Immediately before (0 hours) and 12 hours after the pressure loading, the cell-conditioned buffer was removed and centrifuged. The supernatants were stored at −20°C until renin activity was assayed for determination of RSR. After rinsing with PBS, the cells were frozen in liquid nitrogen and stored at −80°C. For assay of ARC and TRC, frozen cells were homogenized in 1 mL of buffer (pH 6.0) containing (in mmol/L) 2.6 ethylenediaminetetraacetate, 1.6 dimercaprol, 3.4 8-hydroxyquinoline sulfate, 0.2 phenylmethylsulfonfyl fluoride, and 5 ammonium acetate. The homogenates were centrifuged at 12 000 g for 30 minutes, and the supernatant was removed. To measure TRC in JG cells, the samples (900 μL) were incubated at 0°C for 60 minutes with 100 μL of 4 mg/mL trypsin (Sigma Chemical Co., St. Louis, MO) in 500 mmol/L Tris buffer (pH 7.5) containing 5 mmol/L CaCl2, 0.1% NaCl azide, and 1% bovine serum albumin, as described previously.14 Soybean trypsin inhibitor (8 mg/mL) was added to stop the reaction. Thus, inactive renin in the samples was converted to active renin, and renin activity was then determined.

Renin activity was determined as previously described.14 Samples were incubated for 1 hour at 37°C with plasma from bilaterally nephrectomized male Sprague-Dawley rats as the renin substrate, and renin activity was determined by the generation of Ang I levels were measured using a radioimmunoassay coated-head kit from Dinabot Radioisotope Institute (Tokyo, Japan). RSR (%) was calculated as the fractional plasma angiotensinogen substrate. Ang I levels were measured using nephrectomized male Sprague-Dawley rats as the renin substrate, with plasma from bilaterally nephrectomized rats as the renin source.

Effects of NCDC on Transmural Pressure Controls of Renin
Figure 1 shows the effects of 12 hours of exposure to transmural pressure on RSR, ARC, and TRC in JG cells incubated with control buffer or buffer containing 200 μmol/L NCDC (†). The experiment was performed with 5 primary cultures. †P<0.05 for AP versus AP. †P<0.05 for NCDC versus control.

Statistical Analysis
Data were analyzed by paired t test. Differences between treatments were assessed by 2-way analysis of variance followed by multiple comparisons using Scheffe’s F test for repeated measures. A value of P<0.05 was considered significant. Data are presented as mean±SEM.

Results
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Figure 1. Effects of 12-hour exposure to atmospheric pressure (AP) and AP+40 mm Hg on RSR, ARC, and TRC in JG cells incubated with control buffer (A) or buffer containing 200 μmol/L NCDC (†). The experiment was performed with 5 primary cultures. †P<0.05 for AP versus AP. †P<0.05 for NCDC versus control.

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buffer–treated cells. Thus, treatment with NCDC prevented the pressure-induced decrease in RSR.

Treatment with NCDC did not influence ARC under atmospheric pressure or the transmural pressure–induced decrease in ARC. Addition of 40-mm Hg transmural pressure significantly decreased ARC in untreated cells (42.8±3.3 and 26.0±3.9 ng of Ang I·h⁻¹·million cells⁻¹ for atmospheric pressure and atmospheric pressure+40 mm Hg, respectively) and cells treated with NCDC (50.8±9.5 and 28.0±5.0 ng of Ang I·h⁻¹·million cells⁻¹ for atmospheric pressure and atmospheric pressure+40 mm Hg, respectively). The ARC decreases were similar in untreated cells and those treated with NCDC.

Neither the addition of transmural pressure nor treatment with NCDC changed TRC in JG cells. In untreated cells, TRC averaged 99.5 and 28.0±3.9 ng of Ang I·h⁻¹·million cells⁻¹ in untreated cells and those treated with NCDC. In untreated cells, TRC similar in untreated cells and those treated with NCDC.

Effects of U73122 and U73343 on Transmural Pressure Controls of Renin

Figure 2 demonstrates the effects of 12 hours of exposure to transmural pressure on RSR, ARC, and TRC in JG cells treated with U73122 or its inactive analog U73343 at 10 μmol/L (n=5 primary cultures). In the cells treated with U73343, addition of 40-mm Hg transmural pressure significantly decreased RSR from 70.8±3.2 to 46.5±4.6%. In the cells treated with U73122, however, addition of 40-mm Hg transmural pressure did not alter RSR (73.9±3.9 and 72.4±3.5% for atmospheric pressure and atmospheric pressure+40 mm Hg, respectively). RSR of the U73122-treated cells was similar to that of the U73343-treated cells in the group of cells conditioned with atmospheric pressure and significantly greater than that of the U73343-treated cells in the group of cells conditioned with atmospheric pressure+40 mm Hg. Thus, treatment with U73122 prevented the pressure-induced decrease in RSR.

In the cells treated with U73343, addition of 40-mm Hg transmural pressure significantly decreased RSR from 70.8±3.2 to 46.5±4.6%. In the cells treated with U73122, addition of 40-mm Hg transmural pressure also decreased RSR from 73.9±3.9 to 31.9±3.3 ng of Ang I·h⁻¹·million cells⁻¹. The pressure-induced decrease in RSR was similar in the cells treated with U73343 and U73122.

TRC in the cells treated with U73343 and U73122 averaged 106.1±6.6 and 107.0±11.1 ng of Ang I·h⁻¹·million cells⁻¹, respectively. Addition of 40-mm Hg transmural pressure did not influence TRC in either cells treated with U73343 (104.8±6.4 ng of Ang I·h⁻¹·million cells⁻¹) or those treated with U73122 (109.4±7.1 ng of Ang I·h⁻¹·million cells⁻¹). Trypan blue exclusion staining indicated respective cell viabilities of 97.8±0.6 and 97.2±0.4% in the atmospheric pressure–loaded cells treated with U73343 and U73122 and respective cell viabilities of 97.1±0.6 and 96.8±0.4% in the RSR of the U73122-treated cells was similar to that of the U73343-treated cells in the group of cells conditioned with atmospheric pressure and significantly greater than that of the U73343-treated cells in the group of cells conditioned with atmospheric pressure+40 mm Hg. Thus, treatment with U73122 prevented the pressure-induced decrease in RSR.

Effects of AEBSF on Transmural Pressure Control of Renin

Figure 3 illustrates the effects of 12 hours of exposure to transmural pressure on RSR, ARC, and TRC in untreated JG cells and cells treated with 100 μmol/L AEBSF (n=5 primary cultures). In untreated control cells, addition of 40-mm Hg transmural pressure significantly reduced RSR from 73.0±1.9 to 48.6±2.0%. In the cells treated with
AEBSF, RSR under atmospheric pressure averaged 68.8±3.4% and was similar to the RSR of untreated cells. Addition of 40-mm Hg transmural pressure also significantly decreased RSR to 43.9±3.5%. The decreases in RSR were similar in untreated cells and cells treated with AEBSF.

ARC in untreated control cells averaged 69.6±9.0 and 38.8±2.4 ng of Ang I·h⁻¹·million cells⁻¹ for atmospheric pressure and atmospheric pressure+40 mm Hg, respectively, such that transmural pressure significantly decreased ARC. Treatment with AEBSF did not influence ARC of the atmospheric pressure-loaded cells (66.4±13.2 ng of Ang I·h⁻¹·million cells⁻¹) but did prevent the pressure-induced decrease in ARC. In cells treated with AEBSF, ARC of the atmospheric pressure+40 mm Hg-loaded cells averaged 59.4±14.4 ng of Ang I·h⁻¹·million cells⁻¹ and was similar to that obtained under atmospheric pressure. In the group of cells conditioned with atmospheric pressure+40 mm Hg, ARC of the AEBSF-treated cells was significantly greater than that of the control buffer–treated cells.

Neither addition of transmural pressure nor treatment with AEBSF changed TRC in JG cells. In untreated cells, TRC averaged 127.4±17.9 ng of Ang I·h⁻¹·million cells⁻¹ and was not altered by addition of 40-mm Hg transmural pressure (120.2±19.0 ng of Ang I·h⁻¹·million cells⁻¹). In cells treated with AEBSF, TRC averaged 115.2±8.4 ng of Ang I·h⁻¹·million cells⁻¹ and was similar to that in untreated cells. Addition of 40-mm Hg transmural pressure did not change TRC (105.6±5.1 ng of Ang I·h⁻¹·million cells⁻¹). In the AEBSF-treated cells, trypan blue exclusion staining indicated cell viabilities of 95.2±1.4 and 95.6±1.3% after 12-hour loads of atmospheric pressure and atmospheric pressure+40 mm Hg, respectively.

Discussion

Chronic transmural pressure loading inhibits renin secretion and suppresses conversion of inactive to active renin in JG cells. We previously demonstrated transmural pressure–mediated inhibition of renin secretion to be dependent on extracellular Ca²⁺ levels and Ca²⁺ channels and that depletion of intracellular Ca²⁺ stores inhibits transmural pressure–induced decreases in RSR and ARC of JG cells. These results suggest that transmural pressure not only stimulates Ca²⁺ influx but also inhibits renin secretion and prorenin processing through an intracellular Ca²⁺ store–dependent mechanism. In the present study, a 12-hour transmural pressure load decreased RSR and ARC but did not influence TRC in JG cells incubated with control buffer, as is consistent with our previous study. The PLC inhibitors NCDC and U73122 inhibited the RSR decrease but did not influence the ARC decrease with a 12-hour transmural pressure load. However, the PLD inhibitor AEBSF prevented the transmural pressure–induced decrease in ARC without affecting transmural pressure control of renin secretion. These results suggest that a 12-hour transmural pressure load inhibits renin secretion through PLC-dependent pathways and prevents prorenin processing to active renin in JG cells through PLD-dependent mechanisms.

Chronic cyclic stretch significantly decreased renin secretion in both JG cells and renin-expressing As4.1 cells. Although acute mechanical stretch caused Ca²⁺ influx in renin-expressing As4.1 cells, chronic mechanical stretch not only causes Ca²⁺ influx but also activates PLC and inositol phosphates and subsequently mobilizes intracellular Ca²⁺ stores. These results suggest that PLC-dependent pathways play an important role in maintenance of the Ca²⁺ response during chronic mechanical stimulation leading to suppression of renin secretion. In addition, our previous study demonstrated that inhibition of renin release by chronic transmural pressure loading is suppressed by removal of Ca²⁺ from extracellular fluids and by an L-type Ca²⁺ channel blocker. These results suggest that chronic transmural pressure loading may initiate a decrease in renin secretion via Ca²⁺ influx and then continue inhibiting renin secretion from JG cells by stimulating PLC activity.

Our previous study demonstrated prorenin processing inhibition by chronic transmural pressure loading to be dependent on intracellular Ca²⁺ stores. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate IP3 and 1,2-diacylglycerol, which promote release of Ca²⁺ from intracellular stores and activation of protein kinase C, respectively. PLC hydrolyzes phosphatidylycholine to choline and phosphatic acid. Phosphatic acid is known to stimulate IP3 generation and the subsequent mobilization of intracellular Ca²⁺. Because activation of either PLC or PLD causes IP3-dependent Ca²⁺ release from intracellular stores, we hypothesized that chronic transmural pressure loading may modulate renin synthesis and secretion in JG cells through activation of PLC and PLD. However, in the present study, the PLD inhibitor AEBSF inhibited pressure control of prorenin processing, whereas PLC inhibitors did not. A recent study demonstrated that Ang II–induced protein synthesis and cell growth, occurring within minutes or hours, depend on PLD stores, we hypothesized that chronic transmural pressure loading may modulate renin synthesis and secretion in JG cells through activation of PLC and PLD. However, in the present study, the PLD inhibitor AEBSF inhibited pressure control of prorenin processing, whereas PLC inhibitors did not. A recent study demonstrated that Ang II–induced protein synthesis and cell growth, occurring within minutes or hours, depend on PLD.

Cyclic nucleotides play an important role in renin synthesis and secretion in JG cells. However, transmural pressure control of renin does not involve cAMP-dependent pathways, because the adenylyl cyclase activator forskolin does not influence transmural pressure control of renin synthesis or secretion. Studies demonstrated that PLD-dependent signaling pathways are involved in NADPH oxidase-dependent superoxide formation and that superoxide has an inhibitory effect on cyclic guanosine monophosphate (GMP) formation in vascular smooth muscle cells. In JG cells, therefore, PLD-dependent pathways may influence active renin synthesis through the modulation of intracellular cyclic GMP levels as a result of superoxide production.

PLD and its lipid product, phosphatidic acid, are required for vesicle transport from the endoplasmic reticulum to the Golgi complex, in which prorenin is processed to active renin. Although the reason that PLD is required for vesicle transportation is unknown, altered transport of prorenin vesicle may account for the PLD-dependent suppression of prorenin processing by transmural pressure. The other possible mechanisms by
which PLD inhibits conversion of inactive to active renin may be because of the regulation of DNA synthesis by phosphatidic acid.26–27 Because a chronic transmural pressure load has no effect on renin mRNA levels, phosphatidic acid may modulate expression of gene related with processing enzyme activities. Additional studies are needed to elucidate the PLC-independent, PLD-dependent signaling pathways that are responsible for transmural pressure control of prorenin processing in JG cells. Because afferent arteriolar smooth muscle cells transform metabolically into JG cells,11,12 the nature of the pressure-induced intracellular signal transduction may be maintained in JG cells. In afferent arterioles of isolated perfused hydronephrotic rat kidneys, increasing pressure gates mechanosensitive cation channels and causes membrane depolarization and activation of voltage-dependent $\text{Ca}^{2+}$ channels.28 In afferent arterioles of the blood-perfused juxtamedullary nephron, U73122 significantly attenuated vasoconstrictor responses to increasing pressure, suggesting that PLC is involved in the afferent arteriolar constriction in response to increasing pressure.10 In addition, P2 purinoceptors have been demonstrated to be involved in pressure-mediated afferent arteriolar constriction,9 leading to ATP mobilizing $\text{Ca}^{2+}$ from intracellular stores in juxtaglomerular vascular smooth muscle cells, including afferent arteriolar smooth muscle cells.30 Furthermore, activation of PLC has been implicated in the ATP-mediated mobilization of $\text{Ca}^{2+}$ from intracellular stores.31 These studies suggest that both $\text{Ca}^{2+}$ influx and PLC-dependent intracellular $\text{Ca}^{2+}$ mobilization play important roles in the regulation of afferent arteriolar diameters in response to pressure loading. Likewise, when combined with our previous study,1 the present results show that $\text{Ca}^{2+}$ influx, intracellular $\text{Ca}^{2+}$ mobilization, PLC, and PLD contribute to the regulation of renin secretion and synthesis in JG cells via pressure loading. In conclusion, at the JG cell level, a 12-hour transmural pressure load decreases RSR and ARC without influencing TRC, suggesting that transmural pressure loading inhibits renin secretion and prorenin processing to active renin in JG cells. Decreases in RSR and ARC with the pressure loading were inhibited by the PLC inhibitors NCDC and U73122 and the PLD inhibitor AEBSF, respectively. Thus, PLC and PLD play important roles in the inhibition of renin secretion and prorenin processing in response to a chronic transmural pressure load.

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Phospholipase D Contributes to Transmural Pressure Control of Prorenin Processing in Juxtaglomerular Cell
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