Role of Phospholipase A₂ Isozymes in Agonist-Mediated Signaling in Proximal Tubular Epithelium

Subash Harwalkar, Chung-Ho Chang, Nickolai O. Dulin, Janice G. Douglas

Abstract—Angiotensin II in proximal tubule epithelium is known to stimulate the release of arachidonic acid after stimulation of phospholipase A₂ (PLA₂) independent of phospholipase C-mediated signaling. Furthermore, an angiotensin II type 2 receptor subtype has been linked to this signaling cascade. We investigated the regulation and differential stimulation of PLA₂ by comparing the PLA₂ activities associated with the membranes and cytosol of rabbit renal proximal tubular epithelial cells after stimulation with angiotensin II, epidermal growth factor, and bradykinin. Both fractions demonstrated PLA₂ activity that was dithiothreitol insensitive, required micromolar concentrations of Ca²⁺ for optimal activity, and was inhibited in a dose-dependent manner by an antisera to a cytosolic PLA₂ with a molecular mass of 85 kD. However, membrane-associated PLA₂ did not demonstrate significant substrate specificity, whereas 1-steroyl-2-[¹⁴C]arachidonylphosphatidyl choline was the preferred substrate for cPLA₂. An antisera generated against mastoparan, a known PLA₂ activator, inhibited membrane— but not cytosol-associated PLA₂ activity. Membrane fractions showed a broad pH range (7.5 to 8.5) for optimal PLA₂ activity, whereas cytosol was maximum at pH 9.5. Angiotensin II stimulated membrane-associated-PLA₂ activity by 88%, whereas bradykinin and epidermal growth factor inhibited activity by 54% and 41%, respectively. The three agonists stimulated cPLA₂. Moreover, angiotensin II—induced activation of membrane-associated-PLA₂ preceded the activation of cPLA₂. These results demonstrate differential localization and regulation of proximal tubular epithelial PLA₂ isozymes, which may determine the pattern of subsequent arachidonic acid metabolism by the cytochrome P450 system. (Hypertension. 1998;31:809-814.)

Key Words: phospholipase A₂ ■ mastoparan ■ angiotensin II ■ mitogen-activated protein kinase ■ bradykinin ■ isozymes ■ endothelium

PLA₂ isozymes as a group hydrolyze the sn-2 fatty acyl ester bond of membrane phospholipids, generating free fatty acids and lysophospholipids. This mechanism of AA release is believed to be a rate-limiting step in the synthesis of a variety of eicosanoids that are critical modulators of transport, vasoactivity, and inflammation. PLA₂ isozymes have been broadly classified into sPLA₂ and cPLA₂ isozymes. The sPLA₂ isozymes have a molecular mass of ~14 to 18 kD and are regulated by Ca²⁺. The cPLA₂ isozymes have been classified as either Ca²⁺ regulated or Ca²⁺ independent. The Ca²⁺—regulated isozymes have a molecular mass of ~85 to 110 kD, whereas the Ca²⁺—independent isozyme has a molecular mass of ~40 kD. There is negligible sequence homology between the secreted and cytosolic PLA₂ isozymes. Membrane-associated PLA₂ isozymes (20 to 45 kD) also have been described; however, they are less well characterized.

A variety of PLA₂ isozymes have been described in renal tissues ranging in molecular mass from 14 to 110 kD. Despite the fact that cPLA₂ has been linked to a variety of agonists in mesangial cells, including vasopressin, PLA₂ isozymes and their regulation have been poorly described in kidney epithelial cells. During anoxic renal injury, both membrane-associated and cytosolic PLA₂ activations occur. An angiotensin II type 2 receptor subtype has been linked to Ca²⁺—independent apically oriented PLA₂ activation in kidney endothelium. Furthermore, Ang II has been demonstrated to release AA from BBMVs independent of PLC, providing more support for the involvement of membrane-associated PLA₂. We reasoned that the initiation of this signaling cascade would involve activation of a membrane-associated PLA₂ followed by the release of AA. We have shown that Ang II and/or AA activates a series of kinases, including MAPK, which are then known to phosphorylate and activate PLA₂ activity in the cytosol.

The aim of the present study was to test the hypothesis that activation of a membrane-associated PLA₂ by Ang II is independent of cPLA₂. By using membrane and cytosolic fractions isolated from the rabbit renal epithelial cells, we demonstrate that the membrane-associated PLA₂ activity may be regulated differently than the activity present in the cytosol. Ang II stimulates membrane-associated PLA₂, whereas other agonists that bind to a kinase receptor (EGF) and a PLC-coupled receptor (BK) inhibit this isozyme. All three agonists stimulate PLA₂ activity associated with the cytosol. This,
coupled with their differences in temporal stimulation and their reactivity toward various antisera, further emphasizes that the membrane-associated PLA2 activity may be regulated differently than that present in the cytosol.

**Methods**

**Cell Isolation** Renal proximal tubule epithelial cells were isolated from male New Zealand White rabbits as described previously. Briefly, the method involves homogenization of the renal cortex and separation of fully dissociated cells on a discontinuous 30% to 60% Percoll gradient. Cells with a density of ≈1.026 g/mL were removed and cultured in Costar tissue culture flasks according to the method of Chung et al. These cells have been described as possessing the usual characteristics of proximal tubular epithelium. The standard growth medium was a 50:50 mixture of Dulbecco's modified Eagle's and Ham's F12 media supplemented with 15 mmol/L HEPES buffer, pH 7.35, 1.2 mg/mL sodium bicarbonate, 192 IU/mL penicillin, 200 μg/mL streptomycin, 5 μg/mL bovine insulin, 5 μg/mL human transferrin, 5 × 10−7 mol/L hydrocortisone, and 5% FBS. Cells from the 1.026 g/mL Percoll fraction had been shown previously to be derived mainly from the renal cortex.20 PE was undetectable with this method in the membrane fraction. Both were below the limit of detectability in cytosol. Hence, corrections are made for total (85.6 nmol/mg of protein) based on these estimated and reported values for PE.

**Subcellular Fractionation** Agonists or a diluent was added to the medium with 0.5% fatty acid–free BSA. The cells were stimulated with the agonists for 10 minutes (unless otherwise stated), and the incubation was terminated by discarding the medium, rinsing three times, and scraping the cells into 1 mL of homogenization buffer consisting of 50 mmol/L HEPES, pH 7.4, 0.25 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/L EGTA, 60 μmol/L leupeptin, 60 μmol/L pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1000 U/mL Trasylol. Cells were homogenized with 30 strokes in a Dounce homogenizer. The homogenate was spun at 280,000g for 1 hour in a Beckman ultracentrifuge with a Ti 65 rotor. The resulting high-speed supernatant was designated as cytosol fraction, and the pellet was designated as the membrane fraction.

**Assay of PLA2 Activity** PLA2 activity was assayed according to a variation of the method of Ballou et al using APC as a substrate unless stated otherwise. Cytosol and membrane fractions from diluted or agonist exposed cells were diluted to achieve identical protein concentrations. Substrates were dried under nitrogen and resuspended in dimethylsulfoxide. Two microliters of APC or another substrate (15 μmol/L) was pipetted into an Eppendorf microcentrifuge tube. Reactions were initiated by the addition of 34 μL of 5 to 25 μg of protein and 3 mmol/L free Ca2+; the reaction mixture was incubated for 30 minutes at 37°C. The reaction was terminated by the addition of 40 μL of ethanol with 2% (v/v) acetic acid and 10% of 5 mg/mL free AA. Released AA was visualized by iodine staining. With this system, free AA migrates near the front (Rf of >0.5), whereas unhydrolyzed phospholipid remains at the origin. The free AA bands were scraped and counted in 5 mL of Bio-safe scintillation fluid (Research Products International Corp). Blank samples were run routinely containing no cell extract. PC was quantified in membranes in agreement with reported values for rat kidney cortical tubules.20 PE was undetectable with this method in the membrane fraction. Both were below the limit of detectability in cytosol. Hence, corrections are made for total (85.6 nmol/mg of protein) based on these estimated and reported values for PE.

**Immunochromelical Inactivation of Enzyme Using Antiserum Raised Against Mastoparan** The membranes were solubilized after incubation with 0.4% octyl-glucopyranoside (final concentration) on ice for 90 minutes. Cytosol was incubated with the detergent as a control. Membranes and cytosol were preincubated with a 1:200 dilution of mastoparan antiserum at 37°C for 15 minutes before the assay for the PLA2 activity.

**SDS-PAGE and Immunoblotting** The cytosolic and membrane fractions were suspended in SDS-sample buffer and electrophoresed in 8% gels (BioRad) at 90 mA for 18 to 20 hours at room temperature. Standard proteins (high molecular mass from BioRad), prestained standards (BioRad), and recombinant cPLA2 (human cPLA2) as positive control (kindly provided by Dr Ruth Kramer, Eli Lilly, Indianapolis, Ind) also were routinely electrophoresed. After electrophoresis, the proteins were transferred at 60 V for 3 hours (BioRad) onto a Millipore polyvinylidene difluoride membrane. The membranes were blocked overnight at 4°C in blocking buffer containing 5% milk. Membranes were then incubated at 4°C with the primary antibodies (rabbit anti-human cPLA2, antisera [690, 13/B061493]; kindly provided by Dr Ruth Kramer) in the blocking solution for 2 hours. After being washed three times (15 minutes each), the membranes were incubated in the secondary antibodies (1:5000 dilution), conjugated with horseradish peroxidase, in the blocking solution at room temperature for 1 hour. They were...
washed five times (15 minutes each) and then developed using enhanced chemiluminescence (ECL Kit; Amersham).

Induction of Polyclonal Antiserum Against Mastoparan
Mastoparan (3 mg) was conjugated to keyhole limpet hemocyanin (10 mg) by the use of glutaraldehyde (21 mmol/L) in the presence of 0.1 mol/L phosphate buffer, \( \text{pH} 7.0 \). The conjugated peptide (65 \( \mu \)g) was dialyzed against the phosphate buffer to remove glutaraldehyde, mixed with an equal volume of complete Freund’s adjuvant, and subcutaneously injected into New Zealand White rabbits. Four weeks later, rabbits received a booster immunization with 32.5 mg of the conjugated peptide in incomplete Freund’s adjuvant. Rabbits then received a booster immunization every 4 weeks, and antisera were collected 2 weeks after the third booster immunization.

Phospholipid Analysis
Total phospholipids were extracted as described previously. Phospholipids were extracted according to the Bligh-Dyer method from the membrane and cytosol fractions. Samples (in duplicate), along with phospholipid standards, were separated on thin-layer chromatographic plates that were coated with silica gel in chloroform/methanol/20% methylamine (60:36:10, vol/vol/vol). The bands corresponding to PC and PE were cut and transferred to borosilicate glass culture tubes. To the separated PC and PE bands we added 40 \( \mu \)L of 10 N H\(_2\)SO\(_4\) and 70% perchloric acid. The samples were heated to 190°C until dry. After cooling, to the samples, 75 mL of water along with 400 mL of 70% perchloric acid. The samples were heated to 190°C until dry.

Materials
Radiolabeled phospholipid substrates, APC, and APE were obtained from Amersham. Silica gel LK6D plates were from Whatman. Protein was measured with the use of a protein kit from BioRad. Other reagents were obtained from Sigma Chemical.

Statistical Analysis
Values are given as mean±SEM. Unpaired Student’s \( t \) test was used for comparison between two groups. All \( n \) values represent the number of experiments conducted with cells from different animals, with each experiment performed in triplicate. All Ca\(^{2+}\) concentrations represent free Ca\(^{2+}\) calculated using the computer program Free Ca (Fabiato). Two-way ANOVA was used for comparison of multiple groups. Statistical significance was considered to be at the level of \( P<.05 \).

Results

Time Course
Previous observations have documented that after the activation of cPLA\(_2\), there is translacation to membranes, whereas in our experiments, cellular fractions were prepared in the presence of EGTA and EDTA, which results in dissociation of the cPLA\(_2\) from the membranes. The PLA\(_2\) activity in our membrane fraction was designated as membrane-associated PLA\(_2\) activity; hence, it was important to determine the temporal relationship to identify the difference between the PLA\(_2\) activities in the membrane and cytosol after stimulation of intact cells with Ang II (Fig 1). Membrane-associated PLA\(_2\) was significantly increased at 5 minutes, after exposure of intact cells to Ang II, whereas cPLA\(_2\) was increased at 10 minutes.

Ca\(^{2+}\) Requirement for Activity of PLA\(_2\) in Membrane and Cytosolic Fractions
We observed that Ca\(^{2+}\) was necessary to optimize the activity of both compartments, with a maximal increase occurring at 1 \( \mu \)mol/L (Fig 2).

pH Optimum
Optimal PLA\(_2\) activity in the cytosol was observed at \( \text{pH} 9.5 \) as described previously, whereas the membrane-associated PLA\(_2\) activity was observed over a broad range with a plateau between \( \text{pH} 7.5 \) and 8.5 (Fig 3).

Substrate Specificity
With the use of 15 \( \mu \)mol/L APC or APE as exogenous substrates, cytosol-associated PLA\(_2\) activity was 25 times more

<table>
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<tr>
<th>Substrate Specificities for PC and PE</th>
<th>Specific Activity, pmol · mg(^{-1}) · min(^{-1})</th>
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<tbody>
<tr>
<td>PC</td>
<td>PE</td>
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<tr>
<td>Cytosol</td>
<td>150.74±3.48 (n=2)</td>
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<tr>
<td>Membrane</td>
<td>7.24±0.82 (n=2)</td>
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active toward APC than toward APE, whereas membrane-associated activity was only 3.2 times more active toward APC than toward APE (Table).

**DTT Sensitivity**

Because reducing agents have been shown to inhibit sPLA2 activity, DTT was used to determine the influence on PLA2 activity in the membrane versus the cytosolic fractions. DTT (1 mmol/L) did not inhibit enzyme activity associated with either membranes or cytosol, consistent with the absence of sulfhydryl bonds (data not shown).

**Immunochemical Modulation of Enzyme Activity**

We used cPLA2 antisera that recognizes an 85-kD cPLA2 to determine whether differential modulation of enzyme activity might occur (Fig 4). At dilutions of 1:10,000, 1:1000, and 1:100, 19 ± 9%, 83 ± 3%, and 80 ± 4% (n = 2) inhibition below basal levels occurred in membrane fraction and 17 ± 2.4%, 75 ± 11%, and 71 ± 9% (n = 2) inhibition occurred in cytosolic fractions. Because these values did not differ significantly, there is a suggestion that these isozymes have a similar epitope, mastoparan, a polypeptide isolated from wasp venom has been demonstrated to stimulate AA release from membrane phospholipids by activating PLA2.22 Polyclonal antiserum generated against the polypeptide mastoparan was also used to determine whether membrane-associated versus cytosolic PLA2 activities would be affected. The antiserum, at dilutions of 1:200, significantly inhibited membrane-associated activity by 67 ± 9% (n = 3, P < .05) below the basal level; in contrast, cytosolic activity was inhibited by only 21 ± 14% (n = 5), which did not differ significantly from basal activity (Fig 5). Preimmune serum was used as control and did not affect either the membrane-associated or the cytosolic PLA2 activity (data not shown). These observations are consistent with differential regulation of PLA2 activity in these cellular compartments.

**Agonist Modulation of PLA2 Isozymes**

Differential agonist-induced modulation was also observed in that Ang II significantly stimulated the membrane-associated PLA2 activity by 88 ± 34% (n = 9, P < .05), whereas bradykinin and EGF inhibited activity by 54 ± 8% (n = 7, P < .05) and 41 ± 19% (n = 2, P < .05), respectively (Fig 6). However, all agonists significantly increased the cytosolic activity: Ang II by 81 ± 13% (n = 10, P < .05), bradykinin by 54 ± 23% (n = 5, P < .05), and EGF by 38 ± 25% (n = 2, P < .008) (Fig 6). Among this group, Ang II is the only agonist that is not linked to inositol-specific PLC in this cell type, suggesting direct stimulation of membrane-associated PLA2 activity.

**Immunoblot Analysis of PLA2**

Phosphorylation of cPLA2 by MAPK and translocation from cytosol to membranes have been associated with enzyme activation.23 Phosphorylation, which shifts PLA2 activity in the cytosol (≈ 100 kD) to a slower migrating isoform,1 was observed with all agonists and was more pronounced with EGF and BK than with Ang II (Fig 7) despite the fact that in our assay, Ang II–stimulated PLA2 activity in both fractions was higher than the activities that resulted from stimulation with either EGF or BK. Furthermore, EGF- and BK–induced phosphorylation of the cytosolic PLA2 activity was also observed in membrane fractions, consistent with translocation from cytosol to membrane (Fig 7). It is of interest that antiserum also identified a higher molecular mass band of ≈ 126 kD in the membrane fraction that was not observed in the cytosol.

**Discussion**

Several PLA2 isoforms have been found in the kidney, including cPLA2 (≈ 40 to 110 kD),10,24 sPLA2 (≈ 14 to 18 kD),25 and mPLA2 (20 to 45 kD).5,12,26 Membrane-associated PLA2 has been described in proximal tubular epithelium, and activity is modulated after Ang II stimulation of BBMVs and after brief anoxic exposure.9,12 A Ca2+-independent plasmalogen-selective...
tive isoform has also been implicated in hypoxic injury to proximal tubular cells.10

The present results document that both membrane-associated and cytosolic PLAr activation is associated with agonist-induced signaling in renal proximal tubular epithelium. Membrane-associated PLAr activity appears to be regulated differently than that in the cytosol. Our assay conditions were designed to detect the “classic” cPLAr, as described by a number of groups, in that homogenizations were performed in Ca2+-free buffer with EGTA.1–6 We verified that all agonists activate cPLAr in renal epithelial cells despite differing properties. EGF, which is linked to a kinase receptor, has been shown previously to activate cPLAr in glomerular mesangium, and the same signaling occurs in epithelial cells.27–30 BK, which has been shown to be linked to inositol-specific PLC,31 also activates PLAr. Our observations document that Ang II also activates the cPLAr but by a unique mechanism and may involve prior activation of a membrane-associated PLAr and release of AA, which we have documented temporally. Moreover, both AA and Ang II have been shown to activate MAPK, which has been linked to phosphorylation of cPLAr.14 Thus, a novel mechanism for MAPK-mediated activation of the PLAr activity in the cytosol is supported by these observations.

It has been shown previously that EGF induces ω-hydroxylysol metabolism of AA, whereas Ang II induces epoxygenase metabolism in proximal tubular epithelium.32 Currently, we observed that Ang II stimulates but BK and EGF inhibit the membrane-associated PLAr activity, whereas all agonists stimulate the cytosolic activity. The mechanism or mechanisms by which EGF and BK inhibit the membrane activity have yet to be determined. It will be interesting to determine whether the topography of different epithelial PLAr isoforms and cytochrome P450 isoforms influence the pattern of downstream metabolism of AA by various agonists.

We performed SDS-PAGE and immunoblotting using antiserum raised against cPLAr to further characterize agonist interactions with the PLAr activities associated with the membrane and cytosol. We observed that BK and EGF caused a complete phosphorylation-induced shift of PLAr in both the membrane and cytosolic fractions. Similar phosphorylation shifts involving the activation of PLAr have been observed in other cell types.12,33 The Ang II effect on phosphorylation of cytosolic PLAr activity was less pronounced, consistent with an alternative mechanism of activation.

We attempted to characterize proximal tubular PLAr isoforms with respect to physical properties for comparison with other PLAr isoforms. Both the epithelial PLAr activities were DTT insensitive, required micromolar concentrations of Ca2+ for optimal activity, and were inhibited in a dose-dependent manner by cPLAr antisera, characteristics shared with the majority of intracellular PLAr isoforms.3 Several differences, in addition to agonist modulation, include the observation that antisera raised against the peptide mastoparan significantly inhibited the membrane-associated PLAr activity by 70% in contrast to a negligible effect on the cytosol. We were unsuccessful in determining the molecular mass of the protein or proteins interacting with this antisera in kidney epithelium; however, in whole kidney, the protein migrates at ~40 kDa on SDS-PAGE (Chung-Ho Chang, unpublished observations). It is possible that the antisera interacts directly with the activity associated with the membrane or, alternatively, with a regulatory protein.

The optimal pH for cPLAr activity is known to be in the alkaline range,3 consistent with 9.5 in our cytosolic fraction. However, membrane fraction demonstrated a broad range, which plateaued between pH 7.5 and 8.5. Thus, the pH at which the membrane-associated PLAr activity was measured failed to optimize cPLAr activity, supporting the involvement of distinct isoforms. Other differences relate to substrate preferences in which the activity in the cytosolic fraction showed a 25-fold higher activity with APC compared with APE. With membranes, there was only 1.6-fold more activity with APE compared with APC as substrate, further reinforcing the hypothesis that two distinct isoenzymes are involved.
Although our results indicate a strong possibility of the existence of two distinct PLA₂ activities, the possibility of the same cytosolic PLA₁ activity existing at the membrane that is differentially regulated by different agonists cannot be completely ruled out without isolation, purification, and cloning of the proteins involved.

Thus, in summary, we documented differential regulation of membrane-associated and cytosol PLA₂ activity by vasoactive hormones and EGF in proximal tubular epithelium. Ang II, a PLC-independent agonist, activates membrane-associated PLA₂ activity and initiates AA metabolism. Activation of the cPLA₂ activity follows this initial signaling. In contrast, BK and EGF activate the cytosol-associated PLA₂ activity independent of this initial signaling. Some biochemical properties differ in these cellular compartments, suggesting that they represent distinct PLA₂ isoforms.

References

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