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Angiotensin II-Mediated Stimulation of Phospholipase D in Rabbit Kidney Proximal Tubule Cells

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The present study was undertaken to demonstrate whether or not angiotensin II activates a phospholipase D in rabbit kidney proximal tubule cells. By measuring the formation of [^{3}H]phosphatidic acid and [^{3}H]phosphatidylethanol, we elucidate the direct stimulation of phospholipase D by angiotensin II. Angiotensin II leads to a rapid increase in [^{3}H]phosphatidic acid and [^{3}H]diacylglycerol, and [^{3}H]phosphatidic acid formation preceeded the formation of [^{3}H]diacylglycerol. This result suggests that some phosphatidic acid seems to be formed directly from phosphatidylcholine by the action of phospholipase D, not from the action of diacylglycerol kinase on the diacylglycerol. In addition, the other mechanisms by which phospholipase D is activated was examined. We have found that phospholipase D was activated by extracellular calcium ion. It has also been shown that angiotensin II may activate phospholipase D through protein kinase C-independent pathway.

Key words : Angiotensin II, Phospholipase D, Protein kinase C, Calcium ionophore, Phosphatidylethanol, Phosphatidic acid

INTRODUCTION

One of the many effects of neurotransmitters and hormones on cells is to increase the hydrolysis of phospholipids. The most widely studied pathway of phospholipid metabolism is the hydrolysis of phosphatidylinositol and its phosphorylated derivatives by phospholipase C, resulting in the formation of inositol phosphates and diacylglycerol (Horwitz *et al.*, 1991). Receptor-dependent turnover of phosphatidylinositol 4,5-bisphosphate, leads to activation of protein kinase C and mobilization of intracellular ca^{+2} (Thompson *et al.*, 1991). It has recently been suggested that phospholipase D (PLD), as well as phosphoinositide-specific phospholipase C, plays an important role as an effector enzyme in the transmembrane-signaling system (Bonser *et al.*, 1991). PLD is activated by a variety of stimulatory agonists in many different cell types (Kobayashi and Kanfer, 1987; Bonser *et al.*, 1989; Gruchalla *et al.*, 1990; Hiroyuki *et al.*, 1992; Liscovitch and Amsterdam, 1989). The major substrate for this enzyme appears to be phosphatidylcholine (PC), which is hydrolysed to phosphatidic acid (PA) and choline. In combined action with PA phosphohydrolase, PLD can supply diacylglycerol (DAG) from PC, a major component of membrane phospholipid, and the PC-derived DAG, as well as phosphoinositide-derived DAG, might activate protein kinase C or serve as precusor for synthesis of neutral lipids. The proportion of diacylglycerol derived by each of these enzymatic pathways may vary according to cell and tissue type (Huang and Cabot, 1990). PA and its metabolite lysophosphatidic acid (LPA) may also serve a second messenger function since it is known to be either a mitogenic, to activate phospholipase A_2 (PLA_2), to mobilize intracellular calcium, or to inhibit adenylate cyclase (Durieux and Lynch, 1993; Boarder *et al.*, 1994; Moolenaar *et al.*, 1986). In vascular smooth muscle cells, vasoconstrictor and hypertrophic agent angiotensin II activates cytosolic PLA_2. PLA_2 may be one of the major components involved in cell signaling and proliferation (Gadiparthi *et al.*, 1994). Angiotensin II also has been shown to stimulate phosphoinositide metabolism in cultured vascular smooth muscle cells (Griendling *et al.*, 1987). More recently, Griendling and his colleagues (1991) reported that angiotensin II induces a PC hydrolysis, mainly via phospholipase D activation. There are increasing evidences that PLD is activated by several other neurotransmitters and autacoids including angio-

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tensin II, but in order to clarify physiological significances of agonist-stimulated phosphatidylcholine breakdown by activation of PLD futher reasearch is clearly required. The ca^{+2} ionophore A23187 increases PLD activity in many cell types (Reinhold *et al.*, 1990; Billah *et al.*, 1989; Horwitz, 1990). These data suggest that the influx of ca^{+2} may be important in the regulation of this enzyme. Finally, phorbol esters have been shown to increase phospholipase D activity in many cell types, indicating that protein kinase C may also be an important factor in the regulation of phospholipase D (Horwitz and Riscanti, 1992).

To figure out an involvement of PLD for the signal transduction pathway of angiotensin II in renal tissue, primary rabbit kidney proximal tubule cells are employed in this study. As mentioned above well known PLD activators such as protein kinase C and calcium ions were also examined whether they showed PLD stimulatory activities or not in the rabbit kidney proximal tubule cells.

MATERIALS AND METHODS

Materials

[^{3}H]Palmitic acid (60 Ci/mmol) was obtained from Du Pont New England Nuclear Reasearch Products (Boston, MA, U.S.A.). Phosphatidylethanol was purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Phosphatidic acid, angiotensin II, 1,2-diolein, calcium ionophore A23187, staurosporine and phorbol 12-myristate 13-acetate (PMA) were all perchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). DME (Dulbecco's Modified Eagles Medium), fetal calf serum and Ham's F12 were obtained from GIBCO (Grand Island, NY, U.S.A.). Growth factors (insulin, hydrocortisone, transferrin), collagenase (class IV), trypsin EDTA, soybean trypsin inhibitor (SBTI), streptomycin, penicillin G, ferrous sulfate, potassium nitrate, sodium phosphate (monobasic and dibasic), glucose, magnesium sulfate, sodium chloride and HEPES were also purchased from Sigma Chemical Company.

Primary Rabbit Kidney Proximal Tubule Cell Culture

Primary rabbit kidney proximal tubule cell cultures were performed by a modification of the method of Chung *et al.* (1982). To summarize, the kidneys of a male white rabbit (2 to 2.5 kg) were perfused via the renal artery, with phosphate buffered saline (PBS), and subsequently with DME/F12 medium and finally 0.5% iron oxide (w/v), such that the kidney turned grey-black in color. Renal cortical slices were homogenized with 4-5 strokes of a sterile Dounced homogenizer, and the homogenate was poured first through a 253 μ and then a 83 μ mesh filter. Tubules and glomeruli on top of the 83 μ filter were transferred into sterile glucose-free modified DME/F12 medium containing a magnetic stirring bar. Glomeruli containing iron oxide were removed with the stirring bar. The remaining purified proximal tubules were briefly incubated in glucose-free modified DME/F12 having 0.125 mg/ml collagenase and 0.025% soybean trypsin inhibitor. The tubule were then washed by centrifugation, resuspended in glucose-free modified DME/F12 containing the three supplements (insulin, hydrocortisone and transferrin) and 1% fetal calf serum (FCS), and transferred into tissue culture dishes. Medium was changed one day after plating and every two or three days thereafter.

Phospholipid Analysis

Rabbit kidney proximal tubule cells were prelabeled for 12-16 h with [^{3}H]palmitic acid (10 μCi/ml) in the culture medium. On the day of the experiment, the cells were washed once with Earle's balanced salt solution (EBS: EBS consists of 116 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 5 mM glucose, and 25 mM HEPES, pH 7.4) and preincubated for 60 min in EBS at 30°C. At this time, the medium was removed, and fresh EBS containing the agents to be tested was added. The incubations were terminated by removing the medium and adding 0.8 ml of methanol/HCl (100 : 6). The lipid were extracted by the method of Andrews and Conn (1987). Cells were scraped from the 30 mm culture dish with a rubber policeman, each mixture was transferred to a glass tube containing 1.6 ml of chloroform, and the dishes were washed with an additional 0.8 ml of methanol/HCl (100 : 6). This mixture was then sonicated for 2 min. To seperate the phases, 0.8 ml of 100 mM KCl was added. The tubes were then shaken vigorously for 1 min and centrifuged for 10 min at 400 g. The lower phase was transferred to a fresh tube, and the aqueous phase was reextracted with another 1.6 ml of chlorform. To the combined organic phase was added 0.8 ml of methanol/1 mM KCl (4 : 3), and this mixture was shaken, and centrifuged as above. The upper phase was discarded, and the organic phase was dried under a stream of nitrogen at room temperature in the hume hood. The phospholipid sample was dissolved in 1 ml of chloroform/methanol (95 : 5), and aliquots of this mixture were taken for thin layer chromatography (TLC) analysis. TLC was done routinely according to Liscovitch (1989) with minor modification. As a mobile phase the upper layer of a mixture consisting of ethyl acetate/isooctane/acetic acid/water (13 : 2 : 3 :710) was used, and on Silica Gel 60 F254 plate (Merck) phosphatidic acid and phosphatidylethanol

standards (2 μg each) were spotted over the sample to allow visualization with iodine. The iodine was allowed to sublimate before scraping, and the TLC scraping were soaked in 0.5 ml of methanol/HCl (100 : 6) and counted by scintillation spectrometry.

Diacylglycerol Analysis

To measure diacylglycerol formation, the cells were labeled and incubated as above. The incubations were terminated by removing the medium and adding 1 ml of methanol. Extractions were done according to the method of Bligh and Dyer (1959). Cells were scraped from the dishes with a rubber policeman, each mixture were added 0.8 ml of 1 M NaCl and 1 ml chloroform, and this mixture was then sonicated for 2 min. To seperate the phases, 1 ml each of chloroform and 1 M NaCl were added. The organic phase was washed twice with 2 ml of 1 M NaCl. Aliquots of this mixture were dried and taken up in chloroform/methanol (95 : 5) for TLC. [^{3}H]Diacylglycerol was isolated by TLC as previously described (Horwitz, 1990). Slica Gel 60 F254 plate was also used in this analysis. The mobile phase was ether/haxane/acetic acid (70 : 30 : 1), and 1,2-diolein (2 μg) was spotted over the sample to allow visualization with iodine. The iodine was allowed to sublimate before scraping and counting.

RESULTS AND DISCUSSION

The Time Course of Angiotensin II-stimulated [^{3}H] lipid Production

Although there is abundant evidence that many hormones and neurotransmitters cause some of their effects through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in their target cells, there are increasing evidences that many of them also stimulate the breakdown of phosphatidylcholine (PC) (Exton, 1990). PC can be hydrolyzed by either phospholipase C or phospholipase D. The expected products of phospholipase C mediated enzymetic reaction are phosphocholine and diacylglycerol, and those for phospholipase D action are choline and phosphatidic acid. These hydrolysis products can be measured by prelabeling the cells with either radiolabeled fatty acids or [^{3}H]choline. While PC has been known to have higher contents of palmitic acid, oleic acid and linoleic acid, PIP_2 is enriched in stearic acid and arachidonic acid (Traynor, 1982). To study the pathways of phosphatidylcholine metabolism, the phospholipids were labeled with [^{3}H]palmitic acid. Fig. 1 shows the time course of the effect of angiotensin II on the formation of [^{3}H]phosphatidic acid and [^{3}H]diacylglycerol. Cells were labeled with [^{3}H]palmitic acid for 12-16 h. On the day of the experiment, the cells were washed once with EBS and preincubated for 60 min at 30°C. The medium was then removed and fresh buffer containing Angiotensin II (1 μM) was added for indicated times. Angiotensin II caused a rapid increase in [^{3}H] phosphatidic acid and [^{3}H]diacylglycerol levels. [^{3}H] Phosphatidic acid was increased significantly and peaked at 1 min. In contrast, the majority of the increase in [^{3}H]diacylglycerol occurred between 1 and 5 min. Thus, [^{3}H]phosphatidic acid formation precedes the peak of [^{3}H]diacylglycerol accumulation. This result suggests that phosphatidic acid is not derived solely from the phosphorylation of diaylglycerol; this is inaccordance with the idea that a portion of the phosphatidic acid may be formed directly from phosphatidylcholine by the action of phspholipase D.

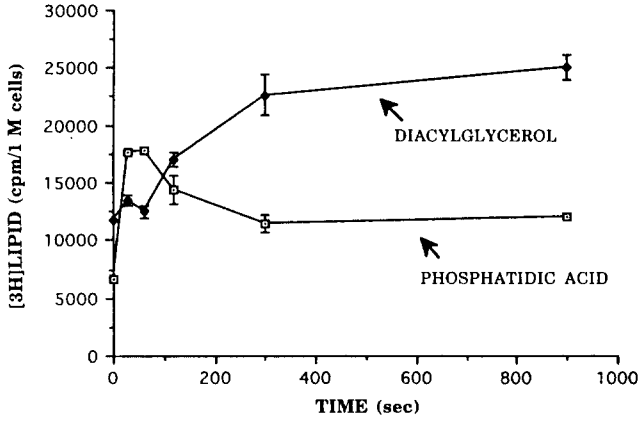

Fig. 1. The time course of angiotensin II-stimulated [^{3}H]lipid formation. Data are mean± SE (bars) values from two independent experiments in triplicate determinations.

The Time Course of Angiotensin II-stimulated [^{3}H] phosphatidic Acid and [^{3}H]phosphatidylethanol Production

Phospholipase D catalyzes not only the hydrolysis of phospholipids, exclusively phosphatidylcholine, but also the transphosphatidylation reaction (Thompson *et al.*, 1991). Phosphatidylethanol is specifically produced by the phosphatidyltransferase activity of phospholipase D in the presence of ethanol, whereas phosphatidic acid, a product by phospholipase D under the physiological condition, is also produced by other pathways (Wang *et al* 1991). Therefore, stimulation of phospholipase D activity in intact cells can be assessed by measuring phosphatidylethanol formation in the presence of ethanol. Fig. 2 shows the time course of angiotensin II-stimulated [^{3}H]phosphatic acid and [^{3}H] phosphatidylethanol formation in rabbit kidney proximal tubule cells in the absence (Fig. 2(A)) or presence

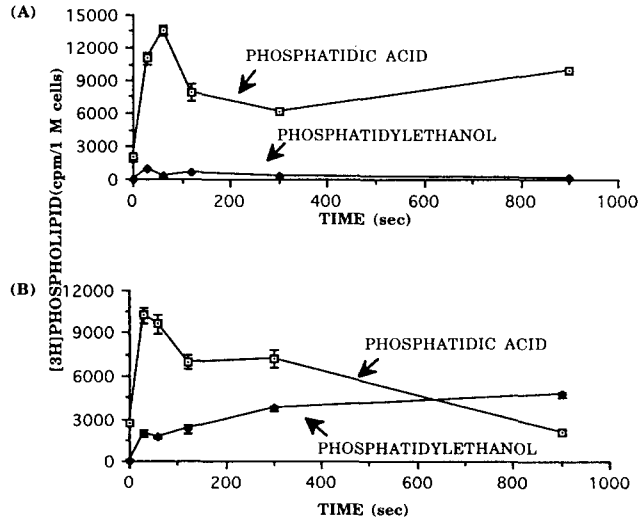

Fig. 2. The time course of angiotensin II-stimulated [^{3}H] phospholipids formation in the absence (a) or presence (b) of 0.5% ethanol. Data are mean± SE (bars) values from two independent experiments in triplicate determinations.

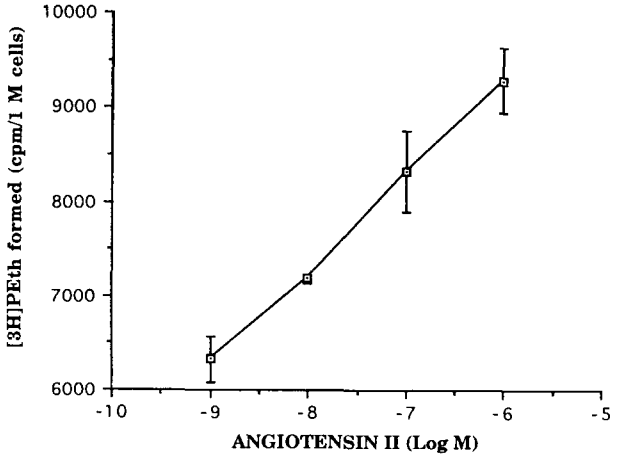

Fig. 3. The concentration dependence of angiotensin II-stimulated [^{3}H]phosphtidylethanol formation. Data are mean ± SE (bars) values from two independent experiments in triplicate determinations.

(Fig. 2(B)) of 0.5% ethanol. Cells were labeled with [^{3}H]palmitic acid for 12-16 h. On the day of the experiment, the cells were washed once with EBS and preincubated for 60 min at 30°C. The medium was then removed and fresh buffer containing angiotensin II (1 μM) with or without 0.5% ethanol was added for indicated times. Angiotensin II induced no formation of phosphatidylethanol in the absence of 0.5% ethanol (Fig. 2(A)). However, in the presence of 0.5% ethanol, angiotensin II induced time dependent accumulation of the phosphatidylethanol, indicating that this agonist is coupled to activation of a phospholipase D. As expected, phosphatidic acid formation is incompletely inhibited by incubation with ethanol, probably

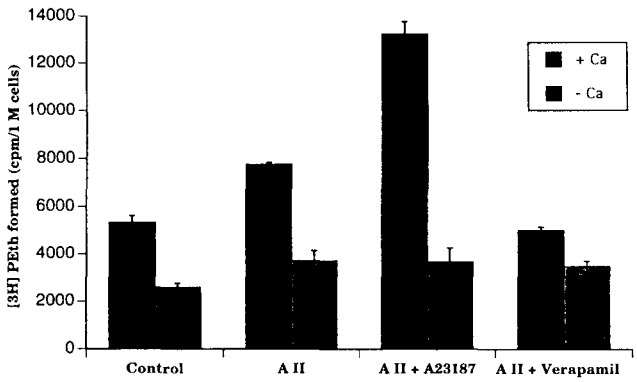

Fig. 4. The ca^{+2} dependence of angiotensin II-stimulated [^{3}H]phosphatidylethanol formation. Data are mean± SE (bars) values from two independent experiments in triplicate determinations.

reflecting the preferential hydrolysis of phosphatidylcholine by phospholipase D with water rather than ethanol, as well as the generation of phosphatidic acid by sequential activation of phosphoinositide-specific phospholipase C and diacylglycerol kinase.

The Concentration Dependence of Angiotensin II-stimulated [^{3}H]phosphatidylethanol Formation

Fig. 3 shows the concentration dependence of angiotensin II-stimulated [^{3}H]phosphatidylethanol formation. The ability of angiotensin II to induce the appearance of the metabolite products of phosphatidylcholine in the extracellular buffer was concentration dependent. Cells were prelabeled with [^{3}H]palmitic acid for 12-16 h. On the day of the experiment, the cells were washed once with EBS and preincubated for 60 min at 30°C. The medium was then removed and fresh buffer containing the indicated concentration of angiotensin II was added and incubated for 5 min, then terminated by removing the medium. [^{3}H]Phosphatidylethanol were formed apparently by more than 10 nM concentration of angiotensin II.

The ca^{+2} Dependence of Angiotensin II-stimulated [^{3}H]phosphatidylethanol Formation

The ca^{+2} requirement for receptor-mediated phosphatidylcholine hydrolysis may vary among cell types (Billah *et al.*, 1990). For angiotensin II-induced phosphatidylcholine hydrolysis, ca^{+2} influx is necessary, but not sufficient in vascular smooth muscle cells (Griendling *et al.*, 1991). Some agonist, such as bradykinin, is known to increase the influx of extracellular ca^{+2} in PC12 cells (Fasolato *et al.*, 1988). Pai *et al.*, (1988) have shown that phosphatidylethanol formation is dependent on extracellular ca^{+2} in HL-60 cells. Therefore, the calcium ion dependence of angiotensin II-stimulated phospholipase D activity was investigated. We also

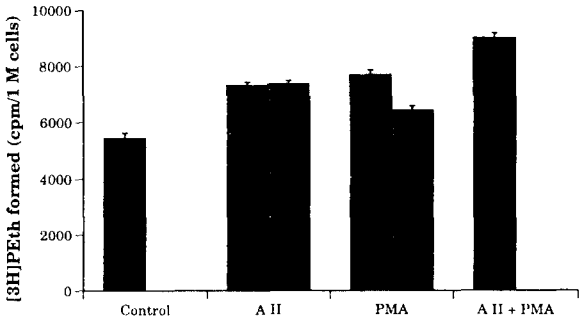

Fig. 5. The protein kinase C dependence of angiotensin II-stimulated [^{3}H]phosphatidylethanol formation. Black bars represent the phosphatidylethanol formation without staurosporine, a nonspecific protein kinase C inhibitor, and those with staurosporine is represented by the grey bars in the figure. Data are mean± SE (bars) values from two independent experiments in triplicate determinations.

like to examine the postulate that activation of phospholipase D in response to angiotensin II is due either solely to an increase of intracellular ca^{+2} by influx of extracellular ca^{+2} or increases of ca^{+2} both intracellular ca^{+2} mobilization and influx from outside. Cells were labeled with [^{3}H]palmitic acid for 12-16 h. On the day of the experiment, the cells were washed once with or without ca^{+2} and then incubated for 5 min in fresh buffer with EBS buffer containing angiotensin II (1 μM/dish) and/or calcium ionophore A23187 (10 μM). Verapamil (10 μM) was added before the expourse to angiotensin II. We incubated proximal tubule cells with excess amounts of EDTA (18.2 mM) before stimulation to deplete both extracellular and intracellular ca^{+2} stores. This treatment, as shown in Fig. 4, was effective in depleting intra- and extracellular ca^{+2} as activation of phospholipase D either by angiotensin II or ca^{+2} ionophore (A23178) was significantly inhibited to the lower than basal level. These results may suggest that angiotensin II induction of phospholipase D proceeds by ca^{+2} dependent pathways. When pretreated with calcium channel blocker verapamil before stimulation by angiotensin II in the absence ca^{+2} ion. [^{3}H]Phosphatidylethanol formation was inhibited to slightly higher than basal level, consisting with idea that angiotensin II increases intracellular ca^{+2} mobilization and calcium influx from outside, since verapamil can only block calcium influx from outside and intracellular ca^{+2} mobilization induced by angiotensin II was unaffected, resulting in more formation of [^{3}H] phosphatidylethanol than basal level. From these data we may conclude that at least part of the effect of angiotensin II on phospholipase D activity is mediated by the influx of extracellular ca^{+2}.

The Protien Kinase C Dependence of Angiotensin II-stimulated [^{3}H]phosphatidylethanol Formation

Phorbol esters are known to activate phospholipase D in a number of different cell types (Billiah and Anthes *et al.*, 1990). As shown in Fig. 5, phospholipase D was activated both by phorbol 12-myristate 13-acetate (PMA) and angiotensin II to approximately same degree. We next wanted to clarify whether or not activation of protein kinase C was essential for activation of phospholipase D in angiotensin II-stimulated proximal tubule cells by stimulating the cells both with angiotensin II and PMA, and by using staurosporine to inhibit the protein kinase C (Tamaoki *et al.*, 1986). Cells were labeled with [^{3}H]palmitic acid for 12-16 h. On the day of the experiment, the cells were washed and incubated in fresh buffer containing angiotensin II (1 μM/dish) and/or PMA (1 μM/dish). Staurosporine (1 μM/dish) was treated for 60 min and exposed to the same concentration of angiotensin II. Staurosporine inhibited PMA-stimulated [^{3}H]phosphatidylethanol formation by aproximately 60%. In contrast, staurosporine did not affect angiotensin II-stimulated [^{3}H]phosphatidylethanol formation. Angiotensin II-amd PMA-stimulated [^{3}H]phosphatidylethanol production was additive. This data are an additional indication that these two agents stimulate the PLD activity by different mechanisms. All these experimental results imply that although protein kinase C can activate phospholipase D, it probably dose not mediate the stimulatory activity of angiotensin II on phospholipase D in rabbit kidney proximal tubule cells.

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