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Identification of Phosphatidylcholine-Phospholipase D and Activation Mechanisms in Rabbit Kidney Proximal Tubule Cells

Jinho Chung, Joobyung Chae and Sunghyun Chung*

College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea
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Abstract: The present study showed that receptor-mediated activation of rabbit kidney proximal tubule cells by angiotensin II, the Ca^{2+} ionophore A23187, or the protein kinase C activator phorbol myristate acetate (PMA) all stimulated phospholipase D (PLD). This was demonstrated by the increased formation of phosphatidic acid, and in the presence of 0.5% ethanol, phosphatidylethanol (PEt) accumulation. Angiotensin II leads to a rapid increase in phosphatidic acid and diacylglycerol, and phosphatidic acid formation preceeded the formation of diacylglycerol. This result suggests that some phosphatidic acid seems to be formed directly from phosphatidylcholine hydrolyzed by PLD. On the other hand, EGTA substantially attenuated angiotensin II and A23187-induced PEt formation, and when the cells were pretreated with verapamil angiotensin II-induced PLD activation was completely abolished. These results provide the evidence that calcium ion influx is essential for the agonist-induced PLD activation. In addition, staurosporine, an inhibitor of protein kinase C, strongly inhibited PMA-induced PEt formation, but was ineffective on angiotensin II-induced PEt accumulation. GTPγS also stimulates PEt formation in digitonin-permeabilized cells, but pretreatment of the cells with pertussis toxin failed to suppress angiotensin II-induced PEt formation. From these results, we conclude that in the rabbit kidney proximal tubule cells the mechanisms of angiotensin II- and PMA-induced PLD activation are different from each other and mediated via a pertussis toxin-insensitive trimeric G protein.

Key words: angiotensin II, G protein, phospholipase D, protein kinase C, rabbit kidney proximal tubule cell.

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 (PLC), resulting in the formation of inositol phosphates and diacylglycerol (DAG) (Horwitz, 1990). There is increasing evidence that phospholipase D (PLD) also plays an important role as an effector enzyme in the transmembrane-signalling system (Bonser *et al.*, 1991). The major substrate for PLD appears to be phosphatidylcholine (PC), which is hydrolysed to phosphatidic acid (PA) and choline. In early studies (Daniel *et al.*, 1986) evidence was obtained for PC hydrolysis by a PC-specific PLC. The agonist-responsive form(s) of PC-PLC, however, has not yet been identified. An alternate indirect pathway for generation of DAG from PC may involve PLD. A major fraction of DAG formed in agonist-stimulated cells is derived from PC, a major component of membrane phospholipid, by the sequential action of PLD and PA phosphohydrolase which dephosphorylates PA to yield DAG (Exton, 1990; Billah and Anthes, 1990).

PLD activation occurs in a wide variety of agonist-stimulated cells and there is much evidence that PC hydrolysis by PLD or PLC is regulated by G-proteins. This comes from studies utilizing permeabilized cells and isolated plasma membranes, many of which have been reviewed earlier (Exton, 1990; Billah and Anthes, 1990). The extensive literature also indicates that depletion of extracellular and/or intracellular Ca^{2+} results in impairment of agonist-stimulated PC hydrolysis in intact cells (Billah and Anthes, 1990; Huang *et al.*, 1992). Activation of PC breakdown by Ca^{2+} ionophores has also been observed in several cell types (Huang *et al.*, 1992). The addition of phorbol esters to intact cells results in increased activity of PLD in most cell types studied (Exton, 1990; Billah and Anthes, 1990), including NIH/3T3 and Swiss/3T3 mouse fibroblasts.

*To whom correspondence should be addressed.
Tel: 82-2-961-0373, Fax: 82-2-966-3885.

Because protein kinase C (PKC) is regarded as the major receptor for phorbol esters, it was suggested that it is involved in the regulation of PLD activity.

Angiotensin II (ANG II) receptors are present on the brush-border and basolateral membranes of the proximal tubule (Brown and Douglas, 1982; Brown and Douglas, 1983) and mediate potent effects of ANG II on transport and cell growth (Geibel *et al.*, 1990). Rabbit proximal tubule cells and segments have been utilized to study these direct effects of ANG II on proximal tubule function (Geibel *et al.*, 1990). However, the signaling mechanisms of proximal tubule ANG II receptors remain incompletely defined. The present study was designed to examine whether rabbit kidney proximal tubule cell PLD is activated either by angiotensin II, the Ca^{2+} ionophore A23187, or the PKC activator phorbol myristate acetate. Activation was demonstrated by the increased formation of PA and, in the presence of 0.5% ethanol, phosphatidylethanol (PEt). The transphosphatidylation reaction, by which the phosphatidyl group of phospholipids is transferred to a primary alcohol such as ethanol, is catalyzed only by PLD.

Materials and Methods

Chemicals and animals

[^{3}H]Palmitic acid (60 Ci/mmol) and [^{3}H]choline (80 Ci/mmol) were obtained from Du Pont-New England Nuclear (Boston, USA). Phosphatidylethanol standard for TLC was purchased from Avanti Polar Lipids (Birmingham, USA). Phosphatidic acid, angiotensin II, 1,2-diolein, calcium ionophore A23187, staurosporine, phorbol 12-myristate 13-acetate (PMA), insulin, hydrocortisone, transferrin, collagenase (class IV), trypsin EDTA, soybean trypsin inhibitor, streptomycin, penicillin G and HEPES were all purchased from Sigma (St. Louis, USA). DME (Dulbecco's Modified Eagles) medium, fetal calf serum and Ham's F12 were obtained from GIBCO (Grand Island, USA). Earle's balanced salt solution (EBS) consisted 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).

The animals used were male white rabbits weighing about 1.8~2.0 kg and obtained from EuyJungBu Animal Center at Kyungki-Do, Korea.

Cell culture

Primary culture of rabbit kidney proximal tubule cells was carried out by a modification of the method of Chung *et al.* (1982). To summarize, the kidneys of a male white rabbit 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 stir bar. Glomeruli containing iron oxide were removed with the stir bar. The remaining purified proximal tubules were briefly incubated in the culture medium containing 0.125 mg/ml collagenase and 0.025% soybean trypsin inhibitor. The tubule were then washed and resuspended in the medium containing the three supplements (insulin, hydrocortisone, transferrin) and 1% fetal calf serum, and transferred into 30 mm 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 18 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 EBS solution and preincubated for 1 h in EBS at 30℃. At this time, the medium was aspirated, and fresh EBS containing the agent(s) to be tested was added. The incubations were terminated by removing the medium and adding 0.8 ml of methanol/HCl (100 : 6). The lipids were extracted by the method of Andrews and Conn (1987). The phospholipid sample obtained was dissolved in 1 ml of chloroform/methanol (95 : 5), and aliquots of this mixture were taken for TLC analysis. TLC was done rountinely according to Liscovitch (1989) with minor modification.

Diacylglycerol analysis

The proximal tubule cells were labeled and incubated as above to measure diacylglycerol formation. The incubation was stopped by removing the medium and adding 1 ml of methanol. Extraction was performed according to the method of Bligh and Dyer (1959), and [^{3}H]diacylglycerol was isolated by TLC as previously described (Horwitz, 1990).

Choline analysis

The cells were prelabeled with [^{3}H]choline (5 μCi /ml) for 24 h in culture medium and then incubated an additional 24 h in the same medium without the labeled choline. On the day of the experiment, the cells were washed with EBS and incubated as above. The incubations were terminated by adding 1 ml of cold 5% trichloroacetic acid, and [^{3}H]choline metabolites were extracted with water-saturated ether and then isolated by TLC as previously described (Horwitz, 1991).

Statistical analysis

Data were shown as the mean± S.D. Statistical evaluation of the data was performed by Student's t-test for either paired or unpaired observations. Values were considered to be statistically different when P was less than 0.05.

Results

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 can be hydrolyzed by either PLC or PLD. The expected products of the PLD mediated enzymatic action on PC, phosphatic acid and choline, can be measured by prelabeling the cells with either radiolabeled fatty acids or choline. While PC has been known to have higher contents of palmitic acid, oleic acid or linoleic acid, PIP_2 is enriched in stearic acid and arachidonic acid (Traynor *et al.*, 1982). In this study the proximal tubule cells were labeled with [^{3}H]palmitic acid to elucidate the pathways of PC metabolism.

Fig. 1(A) shows the time course of the effect of angiotensin II on the formation of [^{3}H]phosphatidic acid and [^{3}H]diacylglycerol in cells. [^{3}H]phosphatidic acid was rapidly increased and peaked at 1 min. On the other hand, [^{3}H]diacylglycerol was formed at a somewhat slower rate and approached its peak at 5 min. Thus, [^{3}H]phosphatidic acid formation precedes the peak of [^{3}H]diacylglycerol accumulation. This outcome suggests that phosphatidic acid is not derived solely from the phosphorylation of diacylglycerol; this is in accord with the hypothesis that a portion of the phosphatidic acid may be formed directly from PC by the agonist-stimulated PLD.

If angiotensin II does in fact stimulate the PLD-mediated PC hydrolysis, another expected product would be choline. The release of [^{3}H]choline from cells whose phospholipids were prelabeled with [^{3}H]choline was measured. Fig. 1(B) shows the time course of angiotensin II-stimulated [^{3}H]choline release. [^{3}H]choline appeared rapidly and reached the apex at 1 min, then declined quickly. The time course of [^{3}H]choline release was quite similar to that of [^{3}H]phosphatidic acid formation. These results provide the evidence that angiotensin II causes an early activation of PLD.

PLD not only catalyzes the transfer of a phosphatidyl group from PC to water to form phosphatidic acid, it can also catalyze the transfer of a phosphatidyl group to various alcohols to produce phosphatidylalcohol (Kobayashi and Kanfer, 1987). These phosphatidylalcohols can be separated from phosphatidic acid by TLC. For the rest of the experiments in this study, [^{3}H]

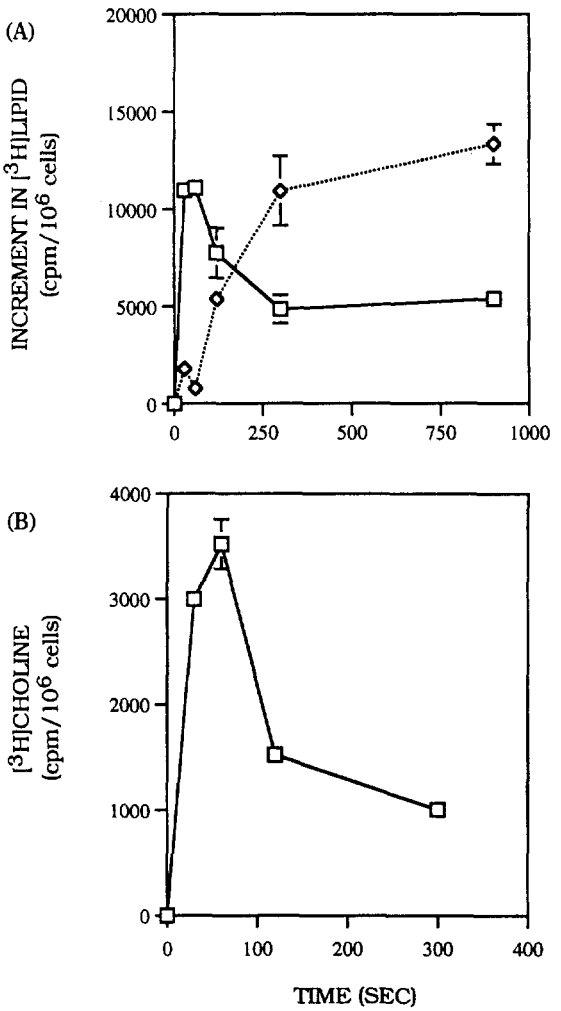

Fig. 1. The time course of angiotensin II-stimulated [^{3}H]phospholipids formation (A) and [^{3}H]choline release (B) in rabbit kidney proximal tubule cells. (A) The time course of angiotensin II-stimulated [^{3}H]phosphatidic acid (solid line) and [^{3}H]diacylglycerol (dotted line) formation in rabbit kidney proximal tubule cells. Cells were labeled with [^{3}H]palmitic acid for 18 h. On the day of the experiment, the cells were washed once with EBS and preincubated for 60 min at 30℃. The medium was then removed and fresh buffer containing angiotensin II (1 μM) was added for the time indicated. [^{3}H]phosphatidic acid and [^{3}H]diacylglycerol were determined in separate, but parallel experiments. The basal levels were 6625± 193 and 11691± 873 cpm/10^6 cells for [^{3}H] phosphatidic acid and [^{3}H]diacylglycerol, respectively. Data points are the average± S.D. of triplicate determinations. (B) The time course of angiotensin II-stimulated [^{3}H]choline release in rabbit kidney proximal tubule cells. Cells were labeled with [^{3}H]choline for 24 h and then incubated for another 24 h in the absence of [^{3}H]choline. On the day of the experiment, the cells were washed once with EBS and preincubated for 60 min at 30℃. The medium was then removed, and fresh medium containing angiotensin II was added for the indicated times.

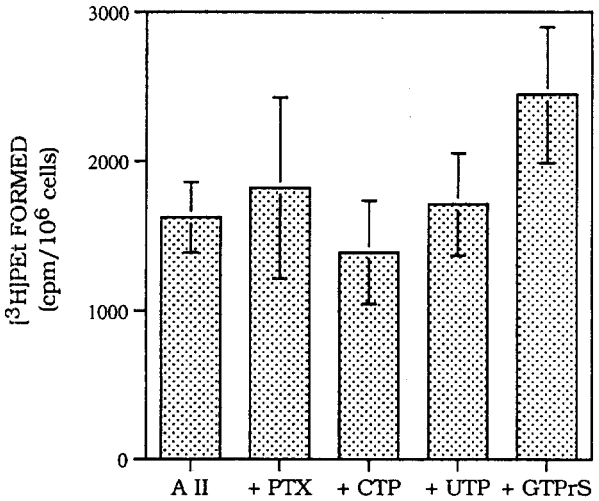

Fig. 2. Effects of various nucleotides including GTPγS and pertussis toxin (PTX) on [^{3}H]PEt formation induced by angiotensin II (A II). To allow the cells to uptake the nucleotides the labeled cells were permeabilized by incubating with 15 μM digitonin in EBS buffer for 6 min at 30℃ prior to stimulation by angiotensin II without or with nucleotides (100 μM for each). The basal level was 4500± 345 for [^{3}H]PEt. To examine whether pertussis toxin exerts inhibitory effect on angiotensin II-induced [^{3}H]PEt formation, the cells were treated with pertussis toxin (2 μM) during prelabeling with [^{3}H]palmitic acid for 18 h before being exposed to angiotensin II. Data points are the average± S.D. of triplicate determinations.

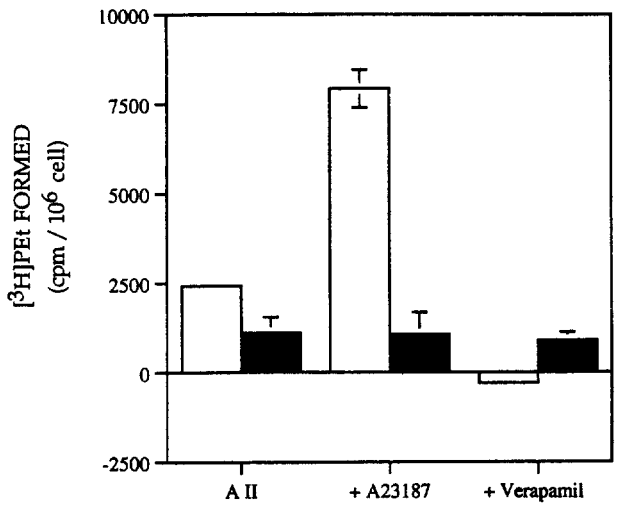

Fig. 3. The Ca^{2+} dependence of angiotensin II-stimulated [^{3}H] PEt formation in rabbit kidney proximal tubule cells. Cells were labeled with [^{3}H]palmitic acid for 18 h, and washed once with EBS buffer with or without Ca^{2+} ion, then incubated in fresh buffer with or without Ca^{2+} (white or black bars, respectively) containing angiotensin II alone or with calcium ionophore A23 187 (1 μM). To examine the effect of calcium channel blocker on the formation of [^{3}H]PEt stimulated by angiotensin II, verapamil (1 μM) was added to the medium 30 min before being exposed to angiotensin II. The basal levels for [^{3}H]PEt formation were 5333± 291 cpm/10^6 cells in the presence of Ca^{2+} and 2591 ± 176 cpm/10^6 cells in the absence of Ca^{2+} Data points are the average± S.D. of triplicate determinations.

phosphatidylethanol ([^{3}H]PEt) was measured to detect the PLD activity under various experimental conditions.

We next examined the possible involvement of a G protein in PLD activation. As shown in Fig. 2, angiotensin II stimulates the [^{3}H]PEt formation, as expected, in digitonin-permeabilized proximal tubule cells. Addition of GTPγS, a nonhydrolyzable GTP analogue, augmented the angiotensin II stimulation of [^{3}H]PEt formation. In contrast, other nucleotides such as CTP and UTP could not augment the angiotensin II stimulation of [^{3}H]PEt formation. Therefore the effect of GTPγS was specific among nucleotides tested in this study.

If G protein indeed mediates angiotensin II activation of PLD, which form(s) of G protein then plays the role in kidney proximal tubule cells? We examined the possible inhibitory effect of pertussis toxin on angiotensin II activation of PLD. As shown in Fig. 2, pretreatment of tubule cells with pertussis toxin for 15 h failed to suppress the [^{3}H]PEt formation by angiotensin II. This result suggests that a G protein involved in PLD activation in tubule cells appears to be pertussis toxin insensitive.

The Ca^{2+} requirement for agonist-stimulated PC hydrolysis may vary among cell types (Billah and Anthes, 1990). We examined whether activation of PLD by angiotensin II is Ca^{2+}-dependent or -independent pathway. Fig. 3 shows that when the proximal tubule cells were exposed to both angiotensin II and Ca^{2+} ionophore A23187 the [^{3}H]PEt formation was induced significantly, and this stimulation was dramatically, but not completely attenuated in the absence of extracellular Ca^{2+} ions. When influx of extracellular Ca^{2+} was inhibited by verapamil, angiotensin II-stimulated [^{3}H] PEt formation was completely abolished. This data suggest that PLD stimulation by angiotensin II is exclusively dependent upon extracellular Ca^{2+} in rabbit kidney proximal tubule cells.

Fig. 4 shows that a tumor promoter PMA, a potent activator of PKC, also stimulates [^{3}H]PEt formation in the labeled proximal tubule cells. The extent of [^{3}H] PEt formation induced by PMA was quite comparable to that induced by angiotensin II, and the [^{3}H]PEt formation was additive when proximal tubule cells were activated by both agents. Staurosporine, a nonspecific PKC inhibitor, inhibited the PMA-induced [^{3}H]PEt formation by approximately 60% at 1 μM. In contrast, staurosporine was not effective on angiotensin II-stimulated [^{3}H]PEt formation. These results suggest that whereas PMA-induced PLD activation is mediated predominantly by PKC, the angiotensin II-induced activation is independent of PKC.

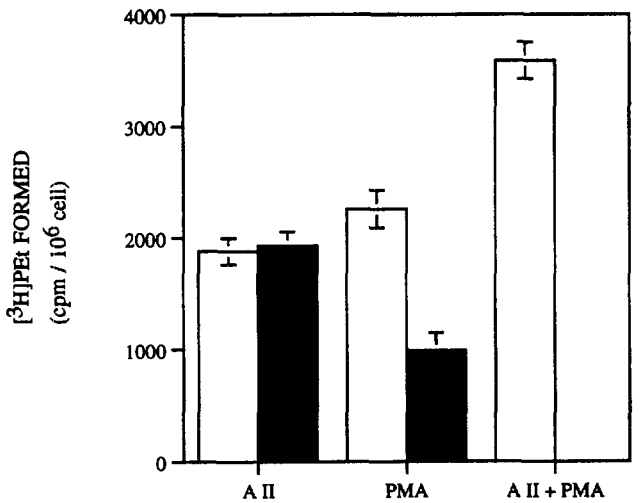

Fig. 4. The protein kinase C dependence of angiotensin II-stimulated [^{3}H]PEt formation in rabbit kidney proximal tubule cells. Cells were labeled with [^{3}H]palmitic acid for 18 h, then washed and incubated in fresh buffer containing either angiotensin II, PMA (100 nM) or both agents. Staurosporine (1 μM) was pretreated for 60 min before being exposed to the same concentration of angiotensin II. Data points are the average± S.D. of triplicate determinations. In this figure white and black bars represent experimental data obtained without and with staurosporine, respectively.

Discussion

In the present study, we have obtained a conclusive evidence that angiotensin II activates PC-PLD in rabbit kidney proximal tubule cells, by showing the formation of [^{3}H]PEt in the presence of ethanol in such cells prelabeled with [^{3}H]palmitic acid. Angiotensin II also increases free [^{3}H]choline in such cells, whose phospholipids have been prelabeled with [^{3}H]choline. The increase in [^{3}H]choline could be produced by a couple of possible pathways. The most likely pathway is the hydrolysis of [^{3}H]PC by PLD. The other possible route is via the action of PLC on PC. This leads to the formation of [^{3}H]phosphocholine which, in turn, can be hydrolyzed to form [^{3}H]choline. The latter pathway, however, is unlikely because several laboratories have shown that [^{3}H]phosphocholine is not degraded to [^{3}H]choline in permeabilized endothelial cells and astrocytes (Martin and Janfer, 1987; Martinson *et al.*, 1989).

The activation of PLD by angiotensin II in proximal tubule cells appears to be mediated by a pertussis toxin-insensitive G protein. This conclusion draws from the findings represented in Fig. 2. The effects of pertussis toxin have not yet been examined extensively. Similar to our observation, inhibition of agonist-stimulated PLD has been reported in neutrophils, HL60 cells and Rat 1 fibroblasts (Kanaho *et al.*, 1992; Xie *et al.*, 1992; MacNulty *et al.*, 1992), while toxin-insensitivity has been observed in PC12 cells and liver plasma membranes (Kanaho *et al.*, 1992; Irving and Exton, 1987).

Activation of PC-PLD by GTP analogues in plasma membranes isolated from some tissues can be observed in the absence of cytosol. Recently two groups, however, independently report the exact same results that the cytosolic factor, known as the low molecular weight GTP-binding protein ARF (ADP-ribosylation factor), is essential for GTPγS-dependent stimulation of PLD in granulocytes and HL60 cells (Cockcraft *et al.*, 1994; Brown *et al.*, 1993).

In addition, we presented evidence that angiotensin II stimulation of PLD activity in proximal tubule cells is independent of PKC, whereas PLD activation by PMA seems to be mediated by PKC. Thus, the mechanism of PLD activation by agonists, like angiotensin II, seems to be distinct from that by phorbol esters in proximal tubule cells. Consistent with our observation, bradykinin-induced PLD activation in PC12 cells is unaffected by staurosporine, although the phorbol dibutyrate-induced activation was effectively blocked (Horwitz and Ricanati, 1990). This is also true in some other types of cells, such as ovarian granulosa cells, LA-N-2 neuroblastoma cells and human neutrophils.

The extensive literature indicating that tumor-promoting phorbol esters stimulate the breakdown of PC to choline or phosphocholine in many cell lines has been reviewed previously. The implication of these studies, that PKC isozymes control PC-PLD and PC-PLC, has been supported by many studies. For example, inhibitors of PKC such as staurosporine, H-7, Ro-31-8220 or sphingosine, block the effects of agonist and phorbol esters, and down-regulation of the enzyme by prolonged phorbol ester treatment has a similar effect. However, it must be recognized that, in many of these studies including ours, the inhibitors caused partial or no impairment of PC-PLD activation by agonists whereas they abolished or substantially reduced the activation by phorbol esters. Since purified PC-PLD is not available to test as a target for PKC isozymes, its mechanism of activation is unknown. In particular, it is not known whether the activation of PLD by PKC is direct or involves an additional protein(s).

Despite its discovery over ten years ago, the physiological significance of agonist or phorbol ester-stimulated PC hydrolysis remains obscure. The activation of PLD by agonists raises two fundamental questions: (a) do the hydrolysis products, PA and choline, generated by the action of PC and PLD play a functional role in proximal tubule cells? and (b) does PA serve as a second messenger or simply as a precursor of either diacylglycerol, lysophosphatidic acid or CDP-diacylglycerol for resynthesis of certain phospholipids?

Although the physiological significance of PC-PLD has recently been reported in several types of cells, e.g. stimulation of superoxide generation in human neutrophils (Rossi *et al.*, 1990), stimulation of insulin release from pancreatic islet cells (Metz and Dunlop, 1990), or supplying choline for acetylcholine synthesis (Hatorri and Kanfer, 1985), more research will be needed to elucidate the role of PC-phospholipase D in kidney proximal tubule cell physiology.

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REFERENCES

Andrew, W. V. and Conn, P. M. (1987) In Methods in Enzymology, Vol. 141 (Means, A. R. and Conn, P. M., eds), pp. 156-168 Academic Press, Orlando, Florida.

Billah, M. M. and Anthes, J. C. (1990) *Biochem. J.* **269**, 281.

Bligh, E. A. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911.

Bonser, R. W., Thompson, N. T., Randall, R. W., Tateson, G. D., Spacey, G. D., Hodson, H. F. and Garland, L. G. (1991) *Br. J. Pharmacol.* **103**, 1237.

Brown, G. P. and Douglas, J. G. (1982) *Endocrinology* **111**, 1830.

Brown, G. P. and Douglas, J. G. (1983) *Endocrinology* **112**, 2007.

Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. and Sternweis, P. C. (1993) *Cell* **75**, 1137.

Chung, S. D., Alavi, N., Livingston, D., Hiller, S. and Taub, M. (1982) *J. Cell Biol.* **95**, 118.

Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O. and Hsuan, J. J. (1994) *Science* **263**, 523.

Daniel, L. W., Waite, M. and Wykle, R. L. (1986) *J. Biol. Chem.* **261**, 9128.

Exton, J. H. (1990) *J. Biol. Chem.* **265**, 1.

Geibel, J., Giebisch, G. and Boron, W. F. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7917.

Hatorri, H. and Kanfer, J. N. (1985) *J. Neurochem.* **45**, 1578.

Horwitz, J. (1990) *J. Neurochem.* **54**, 983.

Horwitz, J. (1991) *J. Neurochem.* **56**, 509.

Horwitz, J. and Ricanati, S. (1990) *Soc. Neurosci. Abstr.* **16**, 370.

Huang, C., Wykle, R. L., Daniel, L. W. and Cabot, M. C. (1992) *J. Biol. Chem.* **267**, 16859.

Huang, R., Kucera, G. L. and Rittenhouse, S. E. (1991) *J. Biol. Chem.* **266**, 1652.

Irving, H. R. and Exton, J. H. (1987) *J. Biol. Chem.* **262**, 3440.

Kanaho, Y., Takahashi, K., Tomita, U., Iiri, T., Katada, T., Ui, M. and Nozawa, Y. (1992) *J. Biol. Chem.* **267**, 23554.

Kanoh, H., Kanaho, Y. and Nozawa, Y. (1992) *J. Neurochem.* **59**, 1785.

Kobayashi, M. and Kanfer, J. N. (1987) *J. Neurochem.* **48**, 1597.

Liscovitch, M. (1989) *J. Biol. Chem.* **264**, 1450.

MacNulty, E. E., McClue, S. T., Carr, I. C., Jess, T., Wakelam, M. J. O. and Miligan, G. (1992) *J. Biol. Chem.* **267**, 2149.

Martin, T. W. and Michaelis, K. (1989) *J. Biol. Chem.* **264**, 8847.

Martinson, E. A., Goldstein, D. and Brown, J. H. (1989) *J. Biol. Chem.* **264**, 14748.

Metz, S. A. and Dunlop, M. (1990) *Biochem. J.* **270**, 427.

Rossi, F., Grzeskowiak, M., Bianca, V. D., Calzetti, F. and Gandini, G. (1990) *Biochem. Biophys. Res. Commun.* **168**, 320.

Traynor, A. E., Schubert, D. and Allen, W. R. (1982) *J. Neurochem.* **39**, 1677.

Xie, M., Jacobs, L. S. and Dubyak, G. R. (1991) *J. Clin. Invest.* **88**, 45.