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# Bradykinin-Mediated Stimulation of Phospholipase D in Rabbit Kidney Proximal Tubule Cells

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**Abstract**—The present study was undertaken to demonstrate whether or not bradykinin activates a phospholipase D in rabbit kidney proximal tubule cells. By measuring the formation of [$^3$H]phosphatidic acid and [$^3$H]phosphatidylethanol we could elucidate the direct stimulation of phospholipase D by bradykinin. Bradykinin leads to a rapid increase in [$^3$H]phosphatidic acid and [$^3$H]diacylglycerol, and [$^3$H]phosphatidic acid formation preceded 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 diacylglycerol by the action of diacylglycerol kinase. In addition, the other mechanisms by which phospholipase D is activated was examined. We have found that phospholipase D was activated and regulated by extracellular calcium ion and pertussis toxin-insensitive G protein, respectively. It has also been shown that bradykinin may activate phospholipase D through protein kinase C-dependent pathway. In conclusion, we are now, for the first time, strongly suggesting that bradykinin-induced activation of phospholipase D in the rabbit kidney proximal tubule cells is mediated by a pertussis toxin-insensitive G protein and is dependent of protein kinase C.

**Keywords** □ bradykinin, phospholipase D, protein kinase C, G protein, calcium ionophore, phosphatidylethanol, phosphatidic acid.

The study of the kallikrein-kinin system began at the turn of the century when it was shown that the intravenous injection of urine caused a pronounced and sustained fall in blood pressure (Abelous and Bardier, 1909). Now we know that urine contains the hypotensive agents kallikrein, kininogen, and kinins, all of renal origin. Despite numerous investigations (Carretero and Scicli, 1980; Levinsky, 1979), the role of the kallikrein-kinin system in the kidney is not yet established. We just suspect that intrarenal kinins may play an important roles in ion and water transport. For example, bradykinin is believed to induce diuresis, natriuresis, and increase renal blood flow with little or no change in glomerular filtration rate (DeFelice and Brousseau, 1988; Stein *et al.*, 1972). The mechanism of these renal actions of bradykinin has not been entirely uncovered.

The kidney is a very rich source of kininases, especially kininase II, which is highly localized in the proximal tubule (Hall *et al.*, 1976). The combined use of captopril and bacitracin, which both can inhibit kininase II effectively, and the availability of tritium- or iodine-labelled bradykinin made the receptor binding study possible. From the receptor binding studies, Tomita (1984) and we (unpublished data) determined that among the nephron segment of rabbit the highest binding was observed in the cortical and medullary collecting tubules. However, specific binding in other cortical segments was also statistically significant.

Activation of $B_2$ type bradykinin receptor, which is known to mediate most physiological effects of bradykinin, leads to stimulation of the activity of phosphatidylinositol-specific phospholipase C (PLC), resulting in the formation of inositol phosphates and diacylglycerol (Portilla *et al.*, 1988), subsequent elevations in intracellular calcium concentration. In addition, in human airway smooth muscle bradykinin-induced increases in intracellular calcium concentration appear to be mediated both by activation of PLC and also $Ca^{+2}$ influx via receptor-operated $Ca^{+2}$ channel (Murray and Kotlikoff, 1991). Kinins may also increase arachidonic acid release and metabolism. Over the years arachidonic

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acid release has been argued to result either from direct activity of phospholipase $A_2$ ($PLA_2$) or from sequential activation of PLC and diacylglycerol lipase (Burch *et al.*, 1990). The calcium release elicited by the inositol phosphates formed in response to PLC activation has been also proposed to secondarily activate $PLA_2$. More recently, phosphatidylcholine-specific PLC and phosphalipase D (PLD) have been demonstrated to be activated by bradykinin (Horwitz, 1991; Van Blitterswijk *et al.*, 1991). There are increasing evidences that PLD is activated by several other neurotransmitters and autacoids including bradykinin, but in order to clarify physiological significances of agonist-stimulated phosphatidylcholine breakdown by activation of PLD more work is clearly required.

To figure out an involvement of PLD for the signal transduction pathway of bradykinin in normal tissue, primary rabbit kidney proximal tubule cells are employed in this study. 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. In addition, many studies have indirectly suggested that $B_2$ bradykinin receptors are coupled to G proteins (Burch and Axelrod, 1987; Ewald *et al.*, 1989), and the recent cloning of a $B_2$ receptor (McEachern *et al.*, 1991) confirms this hypothesis. Thus, we also like to reveal the G protein involvement in bradykinin-mediated signal tranduction through rabbit kidney proximal tubule cell membrane, and to figure out which type of G protein is linked to $B_2$ bradykinin receptor presented on this cell membrane if G protein is believed to be involved in signal transduction pathway.

## Materials and Methods

### Materials

[$^3$H]Palmitic acid (60 Ci/mmol) and [$^3$H]choline chloride (80 Ci/mmol) were obtained from Du Pont New England Nuclear Research Products (Boston, MA, U.S.A.). Phosphatidylethanol was purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Phosphatidic acid, choline, phosphorylcholine, bradykinin, 1,2-diolein, calcium ionophore A23187, GTP$\gamma$S, pertussis toxin, staurosporine and phorbol 12-myristate 13-acetate (PMA) were all purchased 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 Rrabbit 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, first 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 Dounce homogenizer, and the homogenate was poured first through a 253 $\mu$ and then a 83 $\mu$ mesh filter. Tubules and glomeruli on top of the 83 $\mu$ filter were transferred into sterile glucose-free modified DME/F12 medium containing a magnetic stirring bar. Glomeruli contaning 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/m*l* collagenase and 0.025% soybean trypsin inhibitor. The tubules 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 prelabelled for 48 h with [$^3$H]palmitic acid (10 $\mu$Ci/m*l*) 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℃. 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 m*l* of methanol/HCl (100 : 6). The lipids 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 m*l* of chloroform, and the dishes were washed with an additional 0.8 m*l* of methanol/HCl. This mixture was then sonicated for 2 min. To separate the

phases, 0.8 m*l* of 100 mM KCl was added. The tubes were then shaken vigorously for 1 min and centrifuged for 10 min at 400 Hg. The lower phase was transferred to a fresh tube, and the aqueous phase was re-extracted with another 1.6 m*l* of chloroform. To the combined organic phase was added 0.8 m*l* 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 m*l* 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 : 10) was used, and on the Silica Gel 60 F254 plate (Merck) phosphatidic acid and phosphatidylethanol standards (2 $\mu$g 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 m*l* 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 m*l* 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 was transferred to a glass tube, and the dishes were washed with an additional 1 m*l* of methanol. To this mixture were added 0.8 m*l* of 1 M NaCl and 1 m*l* of chloroform, and this mixture was then sonicated for 2 min. To separate the phases, 1 m*l* each of chloroform and 1 M NaCl were added. The organic phase was washed twice with 2 m*l* of 1 M NaCl. Aliquots of this mixture were dried down and taken up in chloroform/methanol (95 : 5) for TLC. [$^3$H]Diacylglycerol was isolated by TLC as previously described (Horwitz, 1990). Silica Gel 60 F254 plate was also used in this analysis. The mobile phase was ether/hexane/acetic acid (70 : 30 : 1), and 1,2-diolein (2 $\mu$g) was spotted over the sample to allow visualization with iodine. The iodine was allowed to sublimate before scraping and counting.

**Choline Analysis**

The cells were prelabelled with [$^3$H]choline chloride (5 $\mu$Ci/m*l*) for 24 h in culture medium and were then incubated an additional 24 h in the same medium without labeled choline. On the day of the experiment, the cells were washed with EBS and incubated as above. The incubations were stopped by removing the medium and adding 1 m*l* of cold 5% trichloroacetic acid. The cells were then allowed to sit for 10 min on ice. The cells were scraped with policeman and washed with 0.5 m*l* of 5% trichloroacetic acid. After centrifugation, the supernatant was extracted four times with water-saturated ether, neutralized with NaOH, and heated to boil off excess ether. [$^3$H]choline metabolites were isolated by TLC. A 100 $\mu$*l* aliquot of the supernatant was dried down under the stream of nitrogen gas, and the sample was taken up in 15 $\mu$*l* of a standard mixture containing choline (10 mg/m*l*) and phosphocholine (20 mg/m*l*) in water. A 10 $\mu$*l* aliquot was spotted on Silica Gel 60 F254 plates. The mobile phase was 0.5% NaCl/ethanol/methanol/ammonia (10 : 6 : 4 : 1). The spots were visualized by exposure to iodine vapors, and the iodine was then allowed to sublimate. [$^3$H]choline-containing metabolites were eluted from the silica gel by soaking in 1 m*l* of 0.1 M NaOH for 4 h (Martin and Michaelis, 1989). After neutralization with 0.1 m*l* of 1.5 M acetic acid, the samples were counted by scintillation spectrometry.

## 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 is increasing evidences that many of them also stimulate the breakdown of phosphatidylcholine (PC) (Exton, 1990). PC can be hydrolyzed by either PLC or PLD. The expected products of PLD action are choline and phosphatidic acid. While PC has been known to have higher contents of palmitic, oleic, and linoleic acids, $PIP_2$ is enriched in stearic acid and arachidonic acid (Traynor *et al.*, 1982). To study the pathways of PC metabolism, the present study was undertaken by labelling the cells with [$^3$H]palmitic acid. According to the Horwitz's experiment, after a 24 h incubation in the presence of [$^3$H]palmitic acid, [$^3$H]phosphatidylcholine accounted for 78 ± 3% of the total label in phospholipids.

Fig. 1 shows the time course of the effect of bradykinin on the formation of [$^3$H]phosphatidic acid and [$^3$H]diacylglycerol in cells prelabelled with [$^3$H]palmitic acid. Bradykinin caused a rapid increase in [$^3$H]-phosphatidic acid and [$^3$H]diacylglycerol levels. [$^3$H]-phosphatidic acid was increased significantly and pea-

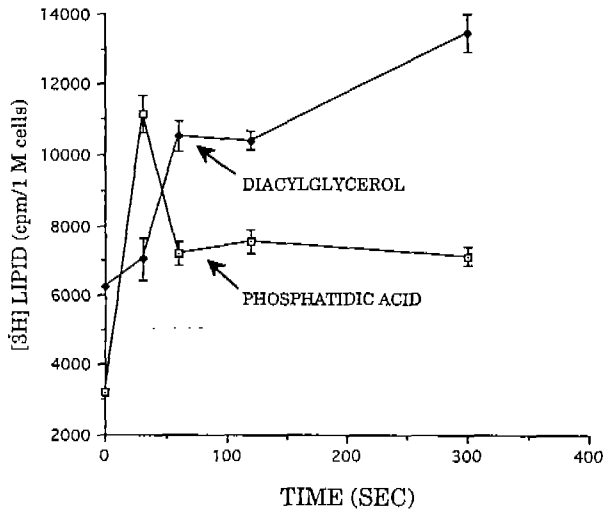


**Fig. 1.** The time course of bradykinin-stimulated [$^3$H]phosphatidic acid and [$^3$H]diacylglycerol production in rabbit kidney proximal tubule cells. Cells were labelled with [$^3$H]palmitic acid for 40~48 h. On the day of the experiment, the cells were washed once with EBS and preincubated for 60 min at 30℃. The medium was then removed and fresh buffer containing bradykinin (20 $\mu$M/30 mm culture dish) 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 3,205 ± 1,128 and 6,220± 893 cpm/10$^6$ cells for [$^3$H]phosphatidic acid and [$^3$H] diacylglycerol, respectively. These lipids were extracted and separated as described in Materials and Methods.

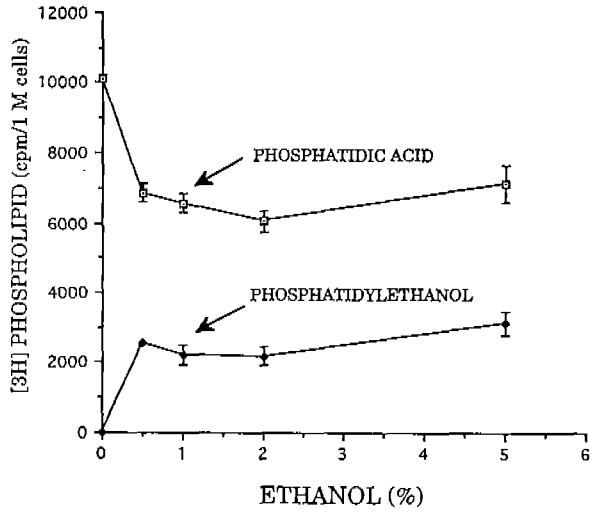


**Fig. 2.** The ethanol dependence of bradykinin-stimulated [$^3$H]phosphatidylethanol formation in rabbit kidney proximal tubule cells. Cells were labelled as before and incubated in the fresh medium containing bradykinin (20 $\mu$M/dish) for 30 sec in the presence of increasing concentration of ethanol (0~5%). The basal levels were 10,109 ± 81 and 1,375 ± 119 cpm/10$^6$ cells for [$^3$H]phosphatidic acid (■) and [$^3$H]phosphatidylethanol (◆), respectively. [$^3$H]phosphatidic acid and [$^3$H]phosphatidylethanol were extracted and separated as described in Materials and Methods.

**Table I.** Bradykinin-stimulated [$^3$H]choline release in rabbit kidney proximal tubule cells

| Metabolite | Control (cpm/10$^6$ cells) | Bradykinin (cpm/10$^6$ cells) |
|---|---|---|
| Choline | 3,667± 599 | 5,333± 549 |
| Phosphocholine | 18,714± 1,403 | 28,238± 1,407 |

Cells were labelled 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 20℃. The medium was then removed, and fresh medium containing bradykinin (20 $\mu$M/dish) was added for 2 min. [$^3$H]Choline metabolites were extracted from the cells and separated by TLC as described in Materials and Methods.

ked at 30 sec. 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 diacylglycerol; this is in accordance with the idea that a portion of the phosphatidic acid may be formed directly from phosphatidylcholine by the action of phospholipase D.

If bradykinin does in fact activate phospholipase D against phosphatidylcholine, another expected reaction product would be choline. The release of [$^3$H]choline from cells whose phospholipids were prelabelled with [$^3$H]choline was also measured. Table I shows the effect of bradykinin on two water soluble metabolites of [$^3$H]choline, choline and phosphocholine. Bradykinin caused a significant increase both in [$^3$H]choline and [$^3$H]phosphocholine. Significant increase in [$^3$H]phosphocholine may result directly from activation of PLC and/or activation of PLD-choline kinase combined pathway. To confirm the observation that bradykinin is a direct stimulator of PLD, the time course of [$^3$H] choline release will be undertaken in the near future.

Fig. 2 shows the effect of ethanol concentration on bradykinin-stimulated [$^3$H]phosphatidylethanol formation. Bradykinin-stimulated [$^3$H]phosphatidyl-ethanol formation was largely dependent upon the presence of ethanol. Formation of [$^3$H]phosphatidylethanol was apparent at 0.5% ethanol. On the other hand, [$^3$H]phosphatidic acid formation was attenuated in the presence of ethanol. This was probably due to the competition between water and ethanol for phosphatidyl group. However, [$^3$H]phosphatidic acid formation was not blocked completely even at high concentrations of ethanol. As shown in Fig. 2, bradykinin-stimulated [$^3$H] phosphatidic acid formation was decreased by only 40

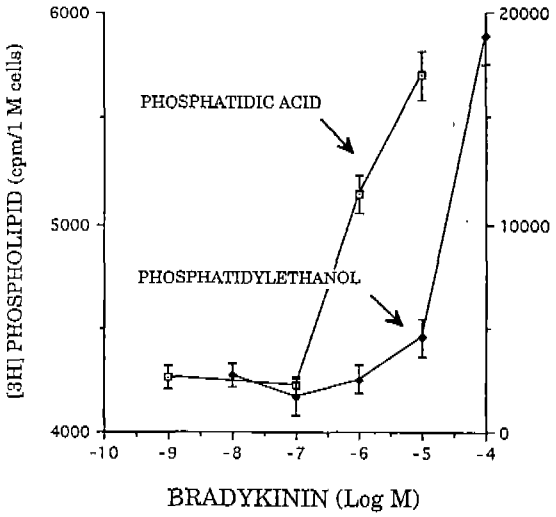


**Fig. 3.** The concentration dependence of bradykinin-stimulated [$^3$H]phosphatidic acid and [$^3$H]phosphatidylethanol in rabbit kidney proximal tubule cells. Cells were labelled with [$^3$H]palmitic acid for 40~48 h. On the day of the experiment, the cells were washed once with EBS and preincubated for 60 min at 30℃. The medium was then removed and fresh buffer containing the indicated concentration of bradykinin was added. The basal levels were 3,789 ± 278 and 1,661 ± 24 cpm/$10^6$ cells for [$^3$H]phosphatidic acid and [$^3$H]phosphatidylethanol, respectively. [$^3$H]phosphatidic acid (▣) and [$^3$H] phosphatidylethanol (◆) were extracted and separated as described in Materials and Methods.

% in the presence of 2% ethanol and even increased slightly at 5% ethanol. This data suggest that [$^3$H]phosphatidic acid is being formed by a different mechanism in addition to the one involving phospholipase D. Subsequent studies were done at 2 min exposure by bradykinin and an ethanol concentration of 5%.

Fig. 3 shows the concentration dependence of bradykinin-stimulated [$^3$H]phosphatidic acid, [$^3$H]phosphatidylethanol formation. These phospholipids were formed apparently by more than 100 nM concentration of bradykinin.

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 bradykinin-stimulated phospholipase D activity was investigated. We also like to examine the postulate that activation of phospholipase D in response to bradykinin is due either solely to an increase of intracellular calcium ion by influx of extracellular calcium ions or increases of calcium ion both by intracellular $Ca^{+2}$ mobilization and influx from ouside. We incubated proximal tubule cells with excess amounts of EDTA (18.2 mM)

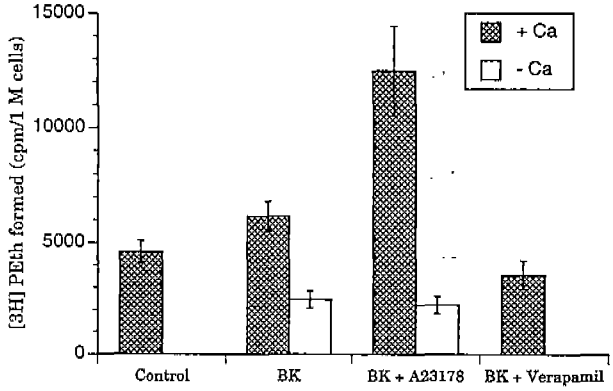


**Fig. 4.** The $Ca^{+2}$ dependence of bradykinin-stimulated [$^3$H]-phosphatidylethanol formation. Cells were labelled with [$^3$H]-palmitic acid for 40~48 h. On the day of the experiment, the cells were washed once with or without $Ca^{+2}$ and then incubated for 2 min in fresh buffer with or without $Ca^{+2}$ containing bradykinin (20 μM/dish) and/or calcium ionophore A23178 (10 μM/dish). Verapamil (10 μM/dish) was added before the exposure of bradykinin. [$^3$H]Phosphatidylethanol was extracted and separated by TLC as described in Materials and Methods.

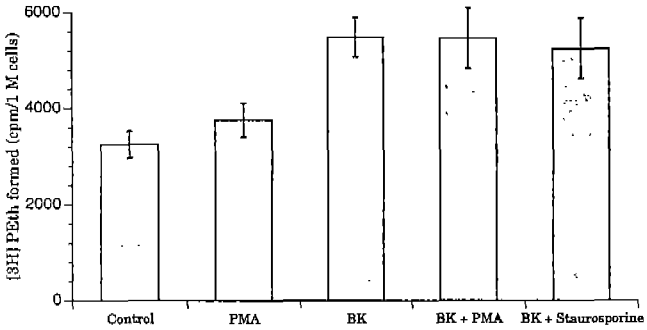


**Fig. 5.** The protein kinase C dependence of bradykinin-stimulated [$^3$H]phosphatidylethanol formation. Cells were labelled with [$^3$H]palmitic acid for 40~48 h. On the day of the experiment, the cells were washed and incubated in fresh buffer containing bradykinin (20 μM/dish) and/or phorbol 12-myristate 13-acetate (PMA, 1 μM/dish). Staurosporine (1 μM /dish) was treated for 60 min and exposed to the same concentration of bradykinin. [$^3$H]Phosphatidylethanol was extracted and separated by TLC as described in Materials and Methods.

before stimulation to deplete both extracellular and intracellular $Ca^{+2}$ stores. This treatment, as shown in Fig. 4, was effective in depleting $Ca^{+2}$ as activation of phospholipase D either by bradykinin or bradykinin and $Ca^{+2}$ ionophore (A23178) was significantly inhibited to the lower than basal level. These results may suggest that bradykinin induction of phospholipase D proceeds by $Ca^{+2}$ dependent pathways. When pretreated with calcium channel blocker verapamil before sti-

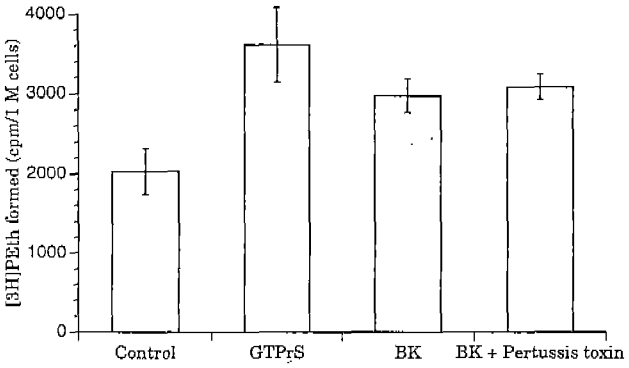


**Fig. 6.** Stimulation of [$^3$H]phosphatidylethanol formation by either GTPγS (100 μM/dish) or bradykinin (20 μM/dish). Cells were labelled with [$^3$H]palmitic acid for 40~48 h. In order to allow GTPγS to get into the cell, the labelled cells were permeabilized by incubating with the digitonin (15 μM/dish) in EBS buffer for 6 min at 30℃. To determine which type of G protein is involved in this intracellular signal transduction pathway, the cells were treated with pertussis toxin in the culture medium for 15 h and then exposed to the bradykinin. [$^3$H]Phosphatidylethanol was extracted and separated by TLC as described in Materials and Methods.

mulation by bradykinin, [$^3$H]phosphatidylethanol formation was inhibited to slightly lower than basal level, consisting with the idea that bradykinin 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 bradykinin was unaffected, resulting in fewer formation of [$^3$H]phosphatidylethanol than basal level. From these data we may conclude that at least part of the effect of bradykinin on phospholipase D activity is mediated by the influx of extracellular $Ca^{+2}$.

Phorbol esters are known to activate phospholipase D in a number of different cell types (Billah and Anthes, 1990). As shown in Fig. 5, phospholipase D was activated by phorbol 12-myristate 13-acetate (PMA), though the level of stimulation was mush less than that induced by bradykinin. We next wanted to clarify whether or not activation of protein kinase C was essential for activation of phospholipase D in bradykinin-stimulated proximal tubule cells by stimulating the cells both with bradykinin and PMA, and by using staurosporine to inhibit the kinase (Tamaoki *et al.*, 1986). Bradykinin- and PMA-stimulated [$^3$H]phosphatidylethanol formation was not additive (bradykinin, 5,469 ± 375; PMA, 3,750 ± 250; bradykinin+PMA, 5,439 ± 512 cpm/$10^6$ cells in 2 min), suggesting that protein kinase C may mediate the effect of bradykinin on phospholipase D. An additional indication that may support the above hypothesis came from the experiments using staurosporine. Staurosporine indeed inhibited bradykinin-induced activation of phospholipase D, though the degree of inhibition was not significant and statistically may not be meaningful.

The involvement of a G protein in phospholipase D activation has been suggested in several types of cells (Bocckino *et al.*, 1987; Xie and Dubyak, 1991). The types of G proteins involved in phospholipase D regulation seem to be different from one type of cell to another. In digitonin-permeabilized proximal tubule cells, GTPγS which is a nonhydrolyzable GTP analogue increased [$^3$H]phosphatidylethanol formation, as shown in Fig. 6. To determine which type of G protein is involved in bradykinin-induced phospholipase D activation, the proximal tubule cells were treated with pertussis toxin for 15 h. Fig. 6 shows that pertussis toxin failed to suppress the [$^3$H]phosphatidylethanol formation by bradykinin, strongly suggesting that bradykinin-induced activation of phospholipase D is, at least in part, mediated by a pertussis toxin-insensitive G protein.

## Discussion

Phospholipase D (PLD) catalyzes not only the hydrolysis of phospholipids, exclusively phosphatidylcholine, but also the transphosphatidylation reaction (Dawson, 1967). Phosphatidylethanol is specifically produced by the phosphatidyl-transferase activity of PLD in the presence of ethanol, whereas phosphatidic acid, a product by PLD under the physiological condition, is also produced by other pathways. Therefore, stimulation of PLD activity in intact cells can be specifically determined by measuring phosphatidylethanol formation in the presence of ethanol.

Bradykinin increases the formation of [$^3$H]phosphatidic acid in the proximal tubule cells prelabelled with [$^3$H]palmitic acid. This result suggests that bradykinin activates a PLD, but this is not conclusive, since [$^3$H] phosphatidic acid can also be formed by the phosphorylation of diacylglycerol and the acylation of glycerol-3-phosphate. PLD has been known to be the only route for phosphatidylethanol formation (Kobayashi and Kanfer, 1987). Accordingly, the fact that bradykinin increases the formation of [$^3$H]phosphatidylethanol in the presence of ethanol confers the conclusive evidence that this autacoid directly activates phospholipase D.

Bradykinin also increases both [$^3$H]choline and [$^3$H] phosphocholine in cells whose phospholipids have been prelabelled with [$^3$H]choline. The likely pathways that cause increase in [$^3$H]choline could be derived from

either direct activation of PLD or sequential actions of phospholipase C on phosphatidylcholine and phosphocholine hydrolase on phosphocholine. The ratio of [$^3$H]phosphocholine to [$^3$H]choline, shown in Table I, suggests that choline is rapidly phosphorylated when it enters the cells. Therefore, it is unlikely that [$^3$H] choline is derived from [$^3$H]phosphocholine. On the other hand, increase in [$^3$H]phosphocholine by bradykinin could also be occurred by two possible pathways. The most likely pathway is the direct activation of PLC acting on phosphatidylcholine. The other possible route is via the action of PLD on phosphatidylcholine. This leads to the formation of [$^3$H]choline which, in turn, can be phosphorylated to form [$^3$H]phosphocholine by choline kinase.

Bradykinin-stimulated [$^3$H]phosphatidic acid formation was not suppressed completely at high ethanol concentrations. Apparently, it is not possible to trap all of the phosphatidyl groups as phosphatidylethanol. This result suggests that phosphatidic acid is derived by other mechanisms besides that involving phospholipase D, namely it is possible that a pool of diacylglycerol is rapidly phosphorylated at short times to form phosphatidic acid. To address this problem, the diacylglycerol kinase inhibitor R 59022 (purchased from Janssen, Olen, Belgium) was employed in our experiment and we observed the significant attenuations of phosphatidic acid formed by bradykinin in the presence of high ethanol concentration (data not shown).

The regulatory mechanisms of agonist control of PLD-induced phosphatidylcholine hydrolysis is still not well understood. Control by either protein kinase C (PKC), calcium ion or G protein has been proposed in several different tissues. Recently, Horwitz and Ricanati demonstrated that activation of protein kinase C leads to an increase in phospholipase D activity and this kinase probably does not play a role in mediating the effect of bradykinin (Horwitz and Ricanati, 1992). However, our results suggesting the existence of a PKC-dependent pathway for the bradykinin-induced PLD activation seem to be different from Horwitz's one, thus the involvement of PKC in PLD activation is still controversial and seems to be dependent on cell types. The relative importance of these regulatory mechanisms remains to be demonstrated.

The real major point we have to consider in the present study is the physiological significance of the activation of phospholipase D by bradykinin. Do the enzymatic reaction product, phosphatidic acid, play a functional role in the cell? Does phosphatidic acid serve as a second messenger or simply as a precursor of diacylglycerol? The functions of phosphatidic acid are presently unknown, but the rapid formation of high concentrations of this lipid during stimulation by agonists strongly suggests that it has signaling functions. Phosphatidic acid is reported to increase cellular calcium concentrations by acting as an ionophore and evoke calcium-mediated physiological responses (Salmon and Honeyman, 1980; Ohsako and Deguchi, 1981). Other study suggests that phosphatidic acid (or lysophosphatidic acid) acts as mitogenic agent (Moolenaar *et al.*, 1986), and this mitogenic effect is totally dependent on c-ras activity (Yu *et al.*, 1988).

Obviously more work will be necessary to elucidate the physiological significance of agonist-activated phospholipase D resulting in hydrolysis of phosphatidylcholine.

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## References

Abelous, J. E. and Bardier, E. (1990). Les substances hypotensives de l'urine humaine normale. *C. R. Soc. Biol.* **66**, 511-512.

Andrews, W. V. and Conn, P. M. (1987). Measurement of inositol phospholipid metabolism by one-dimentional thin-layer chromatography, in *Methods in Enzymology,* Vol. 141 (Means, A. R and Conn, P. M., eds), pp. 156-168. Academic Press, Orlando.

Billah, M. M. and Anthes, J. C. (1990). The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.* **269**, 64-66.

Bligh, E. A. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917.

Bocckino, S. B., Blackmore, P. F., Wilson, P. B. and Exton, J. H. (1987). Phosphatidate accumulation in hormone-treated hepatocytes via a phospholipase D mechanism. *J. Biol. Chem.* **262**, 15309-15315.

Burch, R. M., Farmer, S. G. and Steranka, L. R. (1990). Bradykinin receptor antagonists. *Med. Res. Rev.* **10**, 237-269.

Burch, R. M. and Axelrod, J. (1987). Dissociation of bradykinin-stimulated arachidonic acid release from inositol phosphate formation in Swiss 3T3 fibroblasts. Evidence for a G protein-dependent coupled phospholipase $A_2$. *Proc. Natl. Acad. Sci. USA.* **84**, 6374-6378.

Carretero, O. A. and Scicli, A. G. (1980). The renal kallikrein-kinin system. *Am. J. Physiol.* **238**, F247-F255.

Chung, S. D., Alavi, N., Livingston, D., Hiller, S. and Taub, M. (1982). Characterization of primary rabbit kidney cultures that express proximal tubule functions in a hormonally

defined medium. *J. Cell Biol.* **95**, 118-126.

Dawson, R. M. C. (1967). The formation of phosphatidylglycerol and other phospholipids by the transferase activity of phospholipase D. *Biochem. J.* **102**, 205-210.

DeFelice, A. F. and Brousseau, A. (1988). Natriuretic and vasodilating activities of intrarenally administered atriopeptin II, substance P and bradykinin in the dog. *J. Pharmacol. Exp. Ther.* **246**, 183-188.

Ewald, D. A., Pang, I. H., Sterweis, P. C. and Miller, R. J. (1989). Differential G proteinmediated coupling of neurotransmitter receptors to $Ca^{2+}$ channels in rat dorsal root ganglion neurons *in vitro. Neuron* **2**, 1185-1193.

Exton, J. H. (1990). Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* **265**(1), 1-4.

Fasolato, C., Pandiella, A., Meldolesi, J. and Pozzan, T. (1988). Generation of inositol phosphates, cytosolic $Ca^{+2}$, and ionic fluxes in PC12 cells treated with bradykinin. *J. Biol. Chem.* **263**, 17350-17359.

Hall, E. R., Kato, E. G., Erdos, C. J., Robinson, G. and Oshima, G. (1976). Angiotensin I-converting enzyme in the nephron. *Life Sci.* **18**, 1299-1303.

Horwitz, J. (1990). Carbachol and bradykinin increase the production of diacylglycerol from inositol-containing phospholipids in PC12 cells. *J. Neurochem.* **54**, 983-991.

Horwitz, J. (1991). Bradykinin activates a phospholipase D that hydrolyzes phosphatidylcholine in PC12 cells. *J. Neurochem.* **56**, 509-517.

Horwitz, J. and Ricanati, S. (1992). Bradykinin and phorbol dibutyrate activate phospholipase D in PC12 cells by different mechanisms. *J. Neurochem.* **59**, 1474-1480.

Kobayashi, M. and Kanfer, J. N. (1987). Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D. *J. Neurochem.* **48**, 1597-1603.

Liscovitch, M. (1989). Phosphatidylethanol biosynthesis in ethanol-exposed NG 108-15 neuroblastoma x glioma hybrid cells. *J. Biol. Chem.* **264**, 1450-1456.

Levinsky, N. G. (1979). The renal kallikrein-kinin system. *Circ. Res.* **44**, 441-451.

McEachern, A. E., Shelton, E. R., Bhakta, S., Obernolte, R., and Bach, C. (1991). Expression cloning of a rat $B_2$ receptor. *Proc. Natl. Acad. Sci. USA.* **88**, 7724-7728.

Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J. and de Laat, S. W. (1986). Growth factor-like action of phosphatidic acid. *Nature* **323**, 171-173.

Murray, R. K. and Kotlikoff, M. I. (1991). Receptor-activated calcium influx in human airway smooth muscle cells. *J. Physiol.* **435**, 123-144.

Ohsako, S. and Deguchi, T. (1981). Stimulation by phosphatidic acid of calcium influx and cyclic GMP synthesis in neuroblastoma cells. *J. Biol. Chem.* **256**, 10945-10948.

Pai, J. K., Siegel, M. I., Egan, R. W. and Biliah, M. M. (1988). Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL-60 granulocytes. *J. Biol. Chem.* **263**, 12472-12477.

Portilla, D., Morrissey, J. and Morrison, A. R. (1988). Bradykinin-activated membraneassociated phospholipase C in Madin-Darby canine kidney cells. *J. Clin. Invest.* **81**, 1896-1902.

Salmon, D. M. and Honeyman, T. W. (1980). Proposed mechanism of cholinergic action in smooth muscle. *Nature* **284**, 344-345.

Stein, J. H., Congbalay, R. C., Karsh, D. C., Osgood, R. W. and Ferris, T. F. (1972). The effect of bradykinin on proximal tubular reabsorption in the dog: evidence for functional nephron heterogeneity. *J. Clin. Invest.* **51**, 1709-1721.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/$Ca^{+2}$ dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135**, 397-402.

Tomita, K and Pisano, J. (1984). Binding of [$^3$H]bradykinin in isolated nephron segments of the rabbit. *Am. J. Physiol.* **246**, F732-F737.

Traynor, A. E., Schubert, D. and Allen, W. R. (1982). Alterations of lipid metabolism in response to nerve growth factor. *J. Neurochem.* **39**, 1677-1683.

Van Blitterswijk, W. J., Hilkmann, H., De Widt, J. and Van der Bend, R. L. (1991). Phospholipid metabolism in bradykinin stimulated human fibroblasts. I. Biphasic formation of diacylglycerol from phosphatidylinositol and phosphatidylcholine, controlled by protein kinase C. *J. Biol. Chem.* **266**, 10337-10343.

Xie, M. and Dubyak, G. R. (1991). Guanine nucleotide- and adenine nucleotide dependent regulation of phospholipase D in electropermeabilized HL-60 granulocytes. *Biochem. J.* **278**, 81-89.

Yu, C. L., Tsai, M. H. and Stacey, D. W. (1988). Cellular ras activity and phospholipid metabolism. *Cell* **52**, 63-71.