Biphasic Effects of Exogenous Phospholipase D on Vessel Tone

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Abstract

There exists functional significance for agonists stimulated phospholipase D (PLD) and exogenous PLD in different systems, but the functional importance of PLD on vessel tone is not clear. We studied the functional importance of PLD in whole animals and in isolated vessel preparation. In *in vivo* study, we demonstrated that intravenous injection of PLD at 10, 30, and 100 units to male Sprague-Dawley rats did not significantly alter the mean arterial blood pressure and heart rate in 30 minutes. However, in a denuded vessel preparation, PLD from 10^{-3} units/ml to 10 units/ml induced vasoconstriction from 2.8 ± 1.7 % to 30.5 ± 1.7 % of maximal KCl contraction in calcium enriched physiological salt solution (PSS). This vasoconstrictive effects of PLD were significantly inhibited by omission of extracellular calcium from PSS or by pretreatment the vessels with nifedipine (10^{-6} M). Pretreated the denuded vessels with a protein kinase C inhibitor, chelerythrine (10^{-6} M), did not significantly alter the vasoconstrictive effects of PLD. These results indicate that calcium channel rather than protein kinase C activation is involved in PLD-induced vasoconstriction. In endothelium-intact vessels, application of PLD from 10^{-4} units/ml to 10 units/ml induced endothelium dependent relaxation in vessels precontracted with phenylephrine (10^{-6} M). This relaxation effects of PLD were inhibited by pretreatment of vessels with indomethacin (10^{-5} M) or with N^{ω}-nitro-L-arginine (L-NAME, 10^{-4} M), suggesting prostaglandin and nitric oxide released by PLD stimulation. The biphasic effects of PLD on vessel tone are mediated by extracellular calcium and by endothelium-derived nitric oxide and prostaglandin.

Key Words: phospholipase D, nitric oxide, prostaglandin, protein kinase C, vascular smooth muscle

Introduction

The evidence that mammalian tissues may contain a receptor-linked phospholipase D (PLD) was demonstrated in many tissues (4,13,24). Endogenous PLD activation induces the formation of phosphatidic acid by hydrolysis of phosphatidylcholine (11,19,37). In many tissues, phosphatidate phosphohydrolase inside the tissue can further convert phosphatidic acid to diacylglycerol (14,17). Thus, PLD activation can give rise to diacylglyceride and phosphatic acid as secondary messenger.

Previous studies demonstrated that most agonists that activate PLD also activate the enzyme phospholipase C (PLC) which generates diacylglycerol and inositol trisphosphate (5,35,40). These agonists include angiotensin II (35,40), vasopressin (30), endothelin-1 (35), bradykinin (22), histamine (34), thrombin (26), ATP (5) and biologically active phorbol ester (28). It is hard to differentiate the physiological role between PLD and PLC activation by agonist

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studies, thus PLD may have functional importance in agonist-induced vascular activity.

Studies demonstrated that the activation of endogenous PLD has an important role in DNA synthesis (9,41), protein phosphorylation (2,15), calcium regulation (42), smooth muscle proliferation (32), adenylyl cyclase activation (25,29), phospholipase C activation (31), arachidonic acid releasing (8) and free radical generation (39). The elevation of intracellular arachidonic acid and free calcium by PLD may further induce the formation of prostaglandin and nitric oxide in endothelial cells. Studies also demonstrated that the application of exogenous PLD (from 0.1 to 100 units/ml) increases intracellular calcium (23) and stimulates thymidine incorporation (6) in vascular smooth muscle cells. A previous study showed that the application of exogenous PLD induces cellular proliferation in smooth muscle-like mesangial cells by increasing in intracellular phosphatidic acid and diacylglyceride (21). PLD has effects in many tissues implying that it may play an important role in cellular signal transduction of vascular smooth muscles. The functional significance of PLD on vessel tone is not fully understood. By applying exogenous PLD to isolated vessels and to anesthetized rats, we studied the functional significance of PLD on vessel tone.

Materials and Methods

Adult male Sprague-Dawley rats (250-300 grams) were purchased from National Science Council, ROC. These rats were anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally. For *in vitro* studies, the thoracic cage of the rats was opened by midline incision and thoracic aorta was carefully removed from aortic arch to diaphragm region. The thoracic aortas were dissected free and were immersed in cold physiological salt solution (PSS, 130 mmol/l NaCl, 4.7 mmol/l KCl, 1.18 mmol/l KH_2PO_4, 1.17 mmol/l $MgSO_4$, 1.6 mmol/l $CaCl_2$-$2H_2O$, 14.9 mmol/l $NaHCO_3$, 5.5 mmol/l dextrose, and 0.03 mmol/l $CaNa_2EDTA$). The calcium free PSS solution was prepared by omission of $CaCl_2$-$2H_2O$ and cheleated by 2 mM EGTA. Four aortic rings were cut from each aorta. Aortic rings (4 mm long) were mounted one side to a force transducer (Grass FT03, Quincy, MA) and the other side to a displacement device, so that passive force could be applied (3 g passive force). The aortic rings were placed in jacketed organ baths filled with PSS solution keeping at 37°C and gassed with 95% O_2 and 5 % CO_2 for isometric tension recording throughout the experiment. In some experiments, endothelium was removed by rolling the interior of the ring with a pair of forceps. The removal of endothelium was confirmed by lack of relaxation induced by acetylcholine (10^{-6} M).

After preparation, the vessel segments were allowed to equilibrate for sixty minutes. In endothelium-removed aortic rings, the contractile responses to PLD were studied by applying PLD (from 10^{-4} unit/ml to 10 units/ml) to the bath. In some preparations, the contractile effects of PLD in endothelium-removed vessels were studied by pretreatment the vessels with a protein kinase C inhibitor, chelerythrine (10^{-6} M). To evaluate the functional importance of extracellular calcium in PLD-induced vasoconstriction in denuded vessels, the vessel segments were pretreated with a calcium channel blocker, nifedipine (10^{-6} M).

To study the vasorelaxation effects of PLD, the endothelium-intact or -removed aortic rings were preconstricted with phenylephrine (10^{-6} M). When the contractile responses to phenylephrine reached plateau, PLD from 10^{-4} unit/ml to 10 units/ml was cumulatively applied to the vessels. Previous studies demonstrated that PLD has functional significance at dosage from 0. 1 to 100 units/ml (6,23). In some experiments, the relaxation responses to PLD were studied in endothelium-intact vessels in the presence or absence of indomethacin (10^{-5} M) or N^{ω}-nitro-L-arginine (L-NAME, 10^{-4} M). A previous study demonstrated that the dosage of indomethacin and L-NAME used in our experiment is effective in inhibition of cyclooxygenase and nitric oxide synthase, respectively (16).

The contractile force was measured in mg after exposure of the aortic rings to phospholipase D. The contractile responses to PLD were standardized to percent of the constriction induced by 100 mM KCl and the relaxation responses of PLD were standardized to percent constrictive force induced by 10^{-6} M phenylephrine.

In some preparations, endothelium-intact aortic rings were placed individually in a test tube filling with 0.2 ml physiological salt solution and keeping at 37°C. PLD at concentration of 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 units/ml was added to the physiological salt solution. After 30 minutes, nitrite concentration in the physiological salt solution was measured using NO analyzer (Econ, Japan). This equipment has sensitivity to detect nitrite concentration to the level of 10^{-8} M at the volume of 10 μl.

For blood pressure measurement, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Each rat was placed on a temperature-controlled heated table and the rectal temperature was maintained at 37°C. The trachea was intubated to keep a patent airway and the left femoral vein was catheterized for infusion of an isotonic saline solution (0.01ml/min) and for injection of PLD. The left carotid artery was cannulated with a PE-50 tube for continuous measurement of blood pressure and heart rate was obtained by calculation of the pulsatile pressure signal. All the data were collected and analyzed by PowerLab (ADInstruments).

The following drugs were used for experiments. L-Phenylephrine, nifedipine, indomethacin, acetylcholine, N^{ω}-nitro-L-arginine, and phospholipase D were purchased from Sigma Chemical (St. Louis, MO). Chelerythrine was purchased from Biomol. Res. Lab., Inc. (Plymouth Meeting, PA). Purified PLD from peanut was dissolved in physiological salt solution and was diluted before use. One unit of PLD is defined as the activity that liberates 1 μmol of choline per hour.

Statistics:

Concentration-response curves are expressed as standard error of the means (S.E.M.) for all observations and the concentration of drug is expressed as final organ chamber concentration. Statistical analysis of the data was performed by Student's paired and un-paired observation. A p values less than 0.05 was considered to be statistical significance.

Results

As demonstrated in Figure 1, application of PLD from 10^{-4} units/ml to 10 units/ml significantly caused vasoconstriction in denuded aortic rings. The contractile responses to PLD occurred in a dose-dependent pattern and were significantly inhibited by treating the vessels with a calcium channel blocker, nifedipine (10^{-6} M, Fig. 1, upper panel). The contractile responses to PLD were also significantly inhibited by omission of extracellular calcium (Fig. 1, lower panel). In Figure 2, the vasoconstrictive effects of PLD in denuded aortic rings were not significantly altered by prior incubation of the vessels with a specific protein kinase C inhibitor, chelerythrine (10^{-6} M).

In Figure 3, the application of PLD from 10^{-4} units/ml to 10 units/ml induced dose-dependent relaxation of endothelium-intact aortic rings

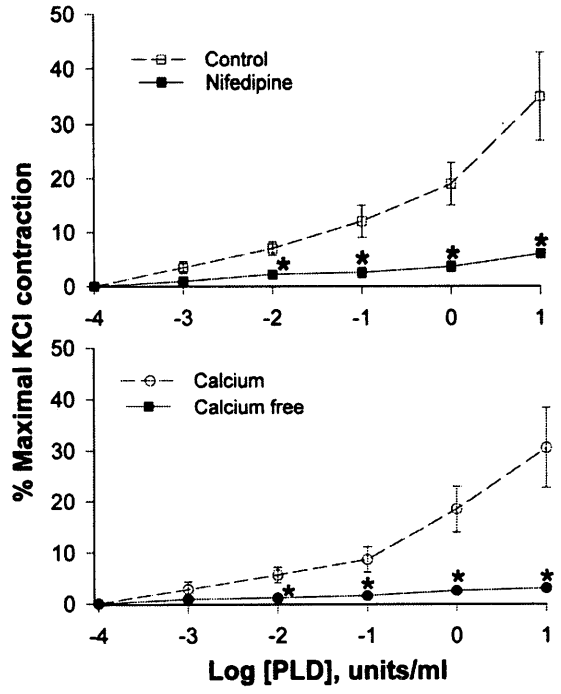

Fig. 1. Contractile responses to phospholipase D (from 10^{-4} units/ml to 10 units/ml) in denuded aortic rings with (closed squares, n = 8) or without (open squares, n = 8) the presence of nifedipine (upper panel), or with (open circles, n = 8) or without (closed circles) the presence of extracellular calcium (lower panel).

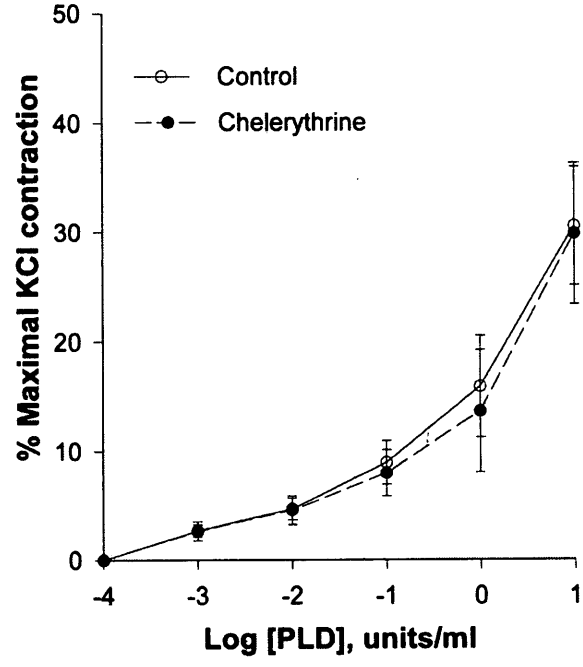

Fig. 2. Constrictive effects of PLD in denuded aortic rings pretreated with (closed circles, n = 6) or without (open circles, n = 6) the presence of chelerythrine (10^{-6} M). Values are mean ± S.E.M.

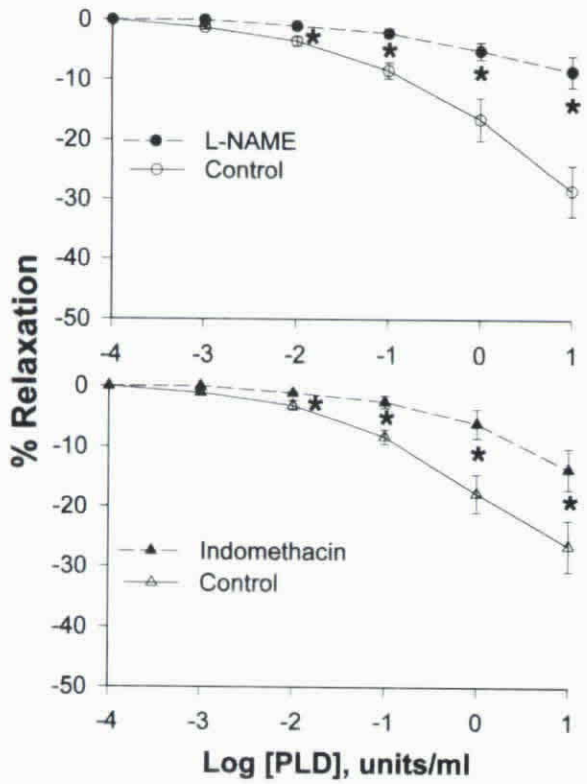

Fig. 3. Relaxation responses to PLD in endothelium-intact aortic rings pretreated with (closed circles, n = 8) or without (open circles, n = 8) the presence of N$^{\omega}$-nitro-L-arginine (L-NAME, 10^{-4} M, upper panel), or with (closed triangles, n = 8) or without (open triangles, n = 8) the presence of indomethacin (10^{-5} M, lower panel). Endothelium intact aortic rings were precontracted with 10^{-6} M phenylephrine. Values are mean ± S.E.M. Asterisks indicate statistically significant difference between groups ($p<0.05$).

precontracted with phenylephrine (10^{-6} M). The relaxation responses to PLD pretreated with or without N$^{\omega}$-nitro-L-arginine (L-NAME, 10^{-4} M) are demonstrated in upper panel of Figure 3. Application of nitric oxide synthase inhibitor, L-NAME, significantly inhibited the relaxation responses to PLD in endothelium-intact aortic rings. Pre-incubation the endothelium-intact vessels with a cyclooxygenase inhibitor, indomethacin (10^{-5} M) also significantly inhibited the relaxation responses to PLD from 10^{-2} units/ml to 10 units/ml (Fig. 3, lower panel). In an isolated vessel preparation, PLD (from 10^{-4} to 10 units/ml) also induced nitrite accumulation in endothelium-intact aortic rings (Figure 4).

The relaxation effects of PLD in the presence or absence of endothelium are demonstrated in Figure 5. The removal of endothelium fully eliminated the relaxation responses induced by PLD.

The mean arterial blood pressure and heart rate responses to intravenous injection of PLD are depicted

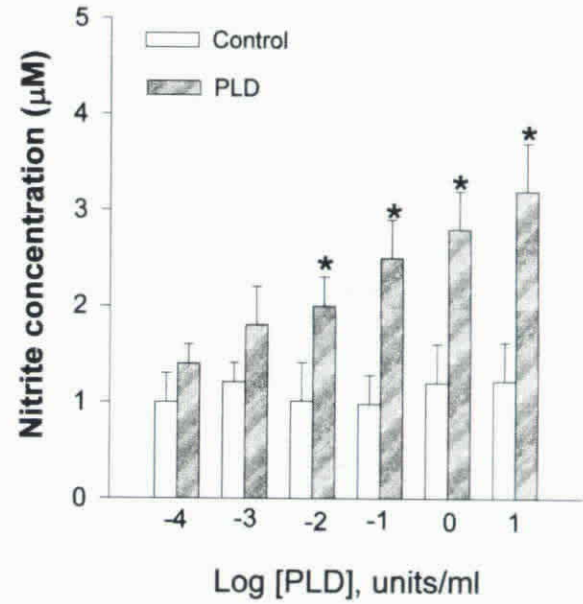

Fig. 4. Nitrite concentration of cendothelium-intact aortic rings incubated in physiological salt solution (0.2 ml) with (open bars) or without (hatched bars) the presence of PLD for thirty minutes. Values are mean ± S.E.M.. Asterisks indicate statistically significant difference between groups ($p<0.05$).

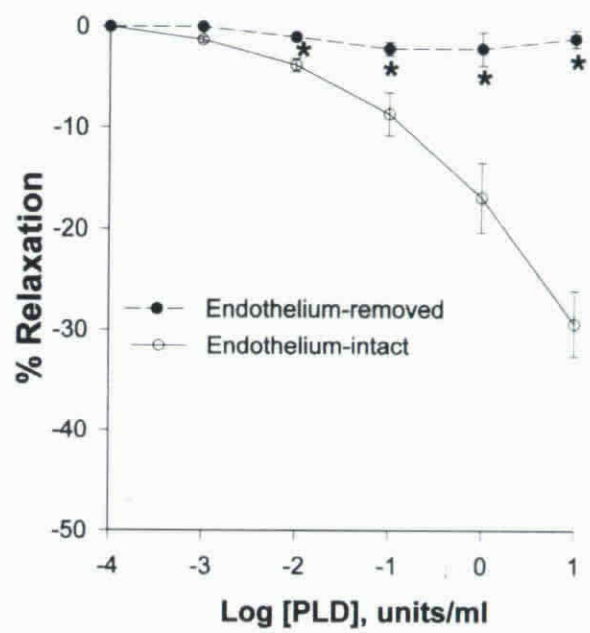

Fig. 5. Relaxation responses to PLD in aortic rings with (open circles, n = 8) or without (closed circles, n = 8) the presence of endothelium. Aortic rings were precontracted with 10^{-6} M phenylephrine. Values are mean ± S.E.M.. Asterisks indicate statistically significant difference between endothelium-removed and endothelium-intact rings ($p<0.05$).

in Figure 6. The mean arterial blood pressure and heart rate at control period were 102 ± 6 mmHg and 394 ± 8 beats/min, respectively. PLD administration at 10, 30,

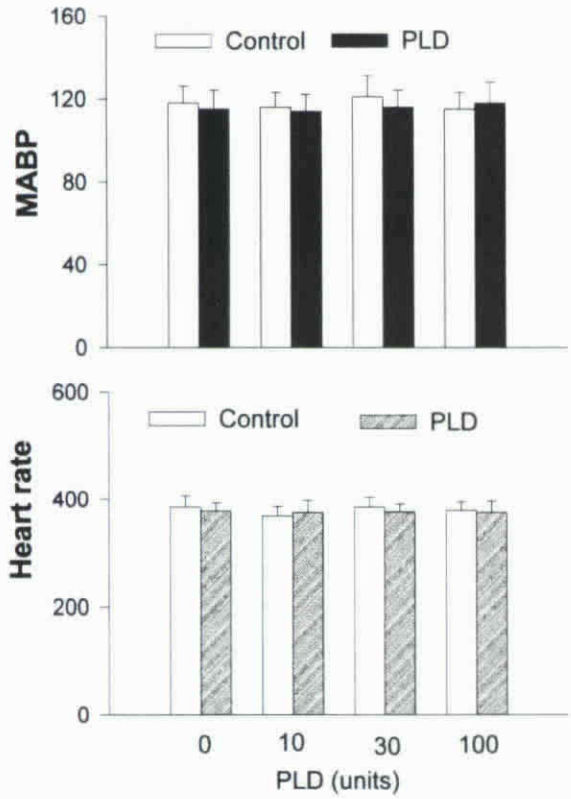

Fig. 6. The mean arterial blood pressure (upper panel) and heart rate (lower panel) responses to intravenous injection of PLD at 10, 30, and 100 units in anesthetized rats (n=6). Values are mean ± S.E.M.

and 100 units did not significantly change the mean arterial blood pressure and heart rate over thirty minute period.

Discussion

Our results demonstrated that exogenous PLD has biphasic effects in regulating vessel tone. Exogenous PLD has been shown to have important functions in stimulation of DNA synthesis (9,41), activation of intracellular calcium (42), and stimulation of insulin release (33). The vasoconstrictive effects of PLD in denuded aortic rings as demonstrated in our results require the presence of extracellular calcium. This vasoconstrictive responses of PLD are mediated by a protein kinase C independent pathway since application of a specific protein kinase C inhibitor, chelerythrine, did not alter the vasoconstrictive responses. The mechanisms for PLD induced vasoconstriction may also be mediated via other mechanisms such as by activation of phosphatic acid (17), by inhibition of adenylate cyclase (29), or by increase the intracellular free calcium (14,42).

Intracellular diacylglyceride and phosphatic acid are interconvertable by a cytoplasmic enzyme, phosphatidate phosphohydrolase (1). A previous study demonstrated that high concentration of propranolol can inhibit the enzyme phosphatidate phosphohydrolase, thus propranolol can help to identify the functional importance between diacylglyceride and phosphatic acid (27). Propranolol inhibited diacylglyceride formation and potentiated the formation of phosphatic acid in vascular smooth muscles (27). Propranolol has been proven to be a useful tool, but the high concentration of propranolol required to inhibit phosphatidate phosphohydrolase is known to have anesthetic effects on nerve (38). We applied propranolol at concentration of 200 mM to block diacylglyceride generation, but propranolol at this concentration fully inhibited the vasoconstrictive responses to 10^{-6} M phenylephrine and 100 mM KCl (data not shown). These effects make propranolol unattractive for studying constrictive responses in vascular smooth muscles. A small number of compounds (demethoxyviridin and wortmannin) have been reported to inhibit endogenous PLD activity by blocking the interaction between receptor and GTP-binding protein in agonists induced PLD activation (10). However, no study ever reported that these compounds have effects on exogenous PLD.

It is possible to question whether the constrictive effects of PLD in denuded vessels are mediated by protein kinase C, since in certain cells, activation of PLD has been suggested to play a role in the activation of protein kinase C (20,31). The other possibility is that the second messenger of PLD, phosphatic acid, can be converted to diacylglyceride that activates protein kinase C and further induces vasoconstriction in denuded vessels. However, pretreatment the endothelium-denuded vessels with a specific protein kinase C inhibitor, chelerythrine, failed to inhibit the vasoconstrictive responses of PLD in denuded vessels. Chelerythrine is a potent and specific inhibitor of protein kinase C with IC_{50} at 0.66 μM (12). We did not observe any significant difference in force generation by PLD after pretreatment the denuded aortic rings with chelerythrine (1μM). Previous studies demonstrated that the activation of PLD by thrombin can convert phosphatidylcholine to diacylglyceride, but this diacylglyceride does not promote the translocation of protein kinase C (28,31). The production of

diacylglyceride from phosphatidylcholine was also not associated with increase phosphorylation of the endogenous substrate for protein kinase C (7,20). Other studies supporting our view are the application of exogenous PLD induces DNA synthesis in vascular smooth muscle cells and this effect is not affected by protein kinase C down regulation (9,18).

Pretreatment of vessels with a calcium channel blocker, nifedipine or with calcium free PSS inhibited the vasoconstrictive effects of PLD suggesting that calcium influx is important in PLD-induced vasoconstriction in endothelium-removed aortic rings. This vasoconstrictive effects of PLD may mediate through the formation of phosphatic acid in vascular smooth muscles since phosphatic acid by itself can induce the influx of extracellular calcium (36,42). Studies in unprimed neutrophils demonstrated that it is the level of phosphatic acid formed from PLD rather than the diacylglyceride levels that correlate with the formyl-Met-Leu-Phen stimulated free radical generation and respiratory burst (3,43). These observations are consistent with our view that phosphatic acid is an important secondary messenger, thus may contribute to PLD-induced vasoconstriction in our observation.

Application of PLD induced vasorelaxation in endothelium-intact aortic rings preconstricted with phenylephrine. The vasorelaxation effects of PLD were dependent on the presence of endothelium since the removal of endothelium eliminated vasorelaxation effects to PLD. Pretreatment of the endothelium-intact vessels with a cyclooxygenase inhibitor, indomethacin or a nitric oxide synthase inhibitor, L-NAME significantly inhibited the relaxation responses to PLD. These results indicate that endothelium-derived prostaglandin and nitric oxide are involved in PLD-induced vasorelaxation. In an isolated vessel (endothelium-intact) preparation, PLD induced nitrite accumulation in a dosage-dependent pattern. This indicates that endothelium-derived nitric oxide is synthesized by PLD stimulation.

In normal vessels, PLD has biphasic effects. The endothelium-dependent vasorelaxation induced by PLD is counteracted by the vasoconstrictive effect of PLD in vascular smooth muscles. Thus, intravenous injection of PLD does not alter the mean arterial blood pressure and heart rate in normal rats as demonstrated by our results. However, in pathological conditions, such as the functional impairment of endothelial cells, PLD may induce abnormal vasoconstriction due to the imbalance between vasoconstrictive and vasorelaxant effects.

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