A hypotensive phospholipid from dog peritoneal dialysate

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The hypotensive effects of lysophosphatidylethanolamines have been reported (Turcotte et al 1973, 1975; Antonello et al 1973). Recently, we have described the vaso-activity of L-α-lysophosphatidic acid (1-acyl-*sn*-glycero-3-phosphate) (Tokumura et al 1978 a,b,c), and the occurrence of an acute depressor-active factor in the acetone-soluble fraction of bovine brain (Tsukatani et al 1976). We now report the pharmacological and chemical characteristics of a short-lasting hypotensive phospholipid obtained from dog peritoneal dialysate (PD) designated tentatively PD.D-I.

Preparation of the hypotensive phospholipid PD.D-I. Dog PD (Martini et al 1967) was lyophilized, extracted with chloroform–methanol (2:1, v/v), washed by Folch's procedure (Folch et al 1957) and the total lipid fraction was obtained (approximately 300 mg litre^{-1} of PD). The lipids were fractionated on a silicic acid column eluted with chloroform–methanol mixtures of increasing polarity, and the phosphorus content in each fraction was determined (Chalvardjian & Rudnicki 1970).

The hypotensive effect of each fraction on carotid arterial blood pressure of the urethane (1·8 g kg^{-1} i.p.)-anaesthetized rat was evaluated by intrafemoral-venous injection of a sample of the eluate after it had been dried and redissolved in 0·9% w/v NaCl. The activity was recovered mainly in the eluate of the chloroform–methanol (4:6, v/v). The active material was further purified through cellulose, first on Sephadex LH-20 [eluting with chloroform–methanol (1:1, v/v)] and second on Sephadex LH-20 [eluting with acetone–ethanol (1:1, v/v)] columns and the hypotensive activities and phosphorus contents of fractions were evaluated as described above. Finally, 1·94 mg of purified hypotensive factor litre^{-1} of PD was obtained which corresponded to approximately 0·65% of the total lipid of dog PD; it contained 92μg of phosphorus. The purified material showed a single spot on t.l.c. in three different solvent systems (Table 1).

Pharmacological properties of PD.D-I. The dose-response relationship (Fig. 1) was examined on urethane-anaesthetized rats.

In the range tested the depressor-response was dose-dependent and no tachyphylaxis or sensitization was observed. The minimum effective dose of PD.D-I was approximately 35·2μg kg^{-1} of the purified preparation on a weight basis and it contained approximately 0·054 μmol phosphate. PD.D-I elicited depressor responses in all species examined approximately to the same extent, but we observed two types of profiles of duration of responses. In rats and cats the arterial blood pressure fell sharply and returned to preinjection values within 2–3 min. In guinea-pigs and rabbits, the blood pressure was reduced more slowly and returned gradually to the initial values; the durations were also dose-dependent. PD.D-I exhibited hypotensive activity even in unanaesthetized rats, although the effects were much less than in anaesthetized animals (pentobarbitone, α-chloralose and urethane). The cardiovascular effect of PD.D-I was not inhibited by pretreatment with antimuscarinic (atropine, 2 mg kg^{-1} i.v.), antihistamine (diphenhydramine, 7 mg kg^{-1} i.v.), β-blocking (propranolol, 1 mg kg^{-1} i.v.) or ganglion-blocking (hexamethonium, 3 mg kg^{-1} i.v.) agents. The hypotensive effect of PD.D-I was also unaffected in the spinal or reserpinized rat. Unlike acetylcholine, PGEs or PGFs, PD.D-I even in large amounts effected no contractions on the guinea-pig isolated ileum preparations. From these results it might be assumed that depressor responses to PD.D-I were not elicited via the central or autonomic nervous system, but by direct actions on certain sites of the cell membrane of the vascular smooth muscle.

Chemical properties of PD.D-I. The purified preparation shows a single spot on t.l.c. with different solvent systems after exposure to iodine vapour or charring with ethanolic sulphuric acid reagent (Table 1). The identity of the depressor activity with the spot was established by preparative t.l.c. with an excellent biological recovery (about 90%) as follows: the preparation was placed on a plate as a streak (Merck silica gel plate 60, 0·25 mm thickness) and DL-lecithin (1,2-dipalmitoyl-glycero-3-phosphocholine), L-α-lysolecithin (1-palmitoyl-*sn*-glycero-3-phosphocholine-LPC) and sphingomyelin were spotted on the other part of the plate as reference compounds. The plate

Table 1. R_F values of the hypotensive factor PD.D-I on t.l.c.

Substance	Solvent (1)	Solvent (2)	Solvent (3)
PD.D-I	0·18	0·29	0·13
PGA_2	0·89	0·97	0·83
PGE_2	0·82	0·93	0·73
DL-Lecithin	0·52	0·86	0·53
L-α-Lysolecithin	0·16	0·28	0·11
Sphingomyelin	0·29	0·61	0·35

Plate: T.l.c. plate silica gel 60(Merck). Detection: iodine vapour.

Solvent (1) chloroform–methanol–acetic acid–water (260:100:4:14, v/v), solvent (2) chloroform–methanol–water (70:30:5, v/v), solvent (3) chloroform–methanol–0·3 N ammonia (230:90:15, v/v). DL-Lecithin (1,2-dipalmitoyl-glycero-3-phosphocholine, Sigma), L-α-lysolecithin(1-palmitoyl-*sn*-glycero-3-phosphocholine, Sigma), sphingomyelin (from bovine brain, Sigma). PGA_2, PGE_2 (Ono Pharmaceutical Industries, Ltd, Japan).

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was developed with chloroform–methanol–water (70:30:5, v/v) at − 20 °C in the dark. Each zone was scraped and extracted with chloroform–methanol (1:1, v/v) and methanol containing acetic acid (0·2 ml in 100 ml) successively. The extracts were combined, decanted, filtered through a glass filter packed with a small amount of hyflosuper-cel and evaporated to dryness for bioassay. The procedure was completed within 6 h at 4 °C.

The purified preparation gave a colour reaction with ethanolic phosphoric acid, antimony trichloride, phosphomolybdate, Dittmer's, Dragendorff's, ferrous hydroxamate, osmic acid or permanganate spray reagents on t.l.c.. Moreover, it was detectable by a spray of 2,4-dinitrophenylhydrazine or 2′,7′-dichlorofluorescein reagent but not to ninhydrin, α-naphthol, diphenylamine, benzidine, mercuric chloride-Schiff's, periodate-Schiff's or blue tetrazolium reagent. A comparison of R_F-values (Table 1) shows PD.D-I to be distinguishable not only from water-soluble hypotensive substances such as acetylcholine, histamine, 5-HT and adenosine derivatives, but also from hypotensive lipids such as PGs or arachidonic acid, although all data are not listed.

In comparison with polar lipids, it strongly resembles LPC, but unlike LPC it had a potent depressor activity and on t.l.c. it is detectable with 2,4-dinitrophenylhydrazine or 2′,7′-dichlorofluorescein reagent. Additionally, a faint spot retarded by sodium bisulphite appeared (relative mobility to L-α-palmitoyl-LPC was about 0·5) on development with chloroform–methanol–5% aqueous sodium bisulphite (70:30:5 v/v) after detection with iodine vapour. The new spot gave a positive reaction with 2,4-dinitrophenylhydrazine reagent.

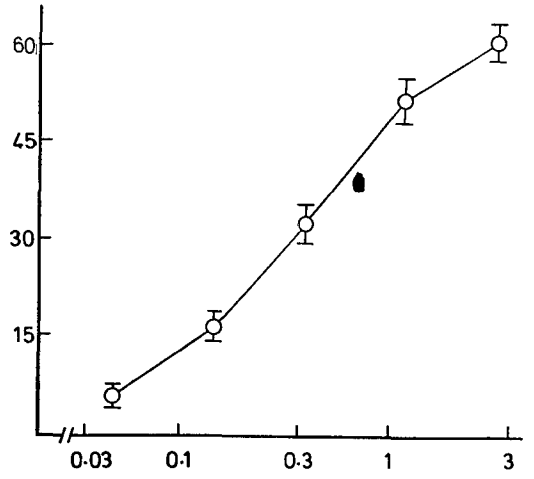

FIG. 1. Dose-response curve for the hypotensive factor PD.D-I. The hypotensive activity of the purified PD.D-I preparation is expressed as a unit value. One unit (U) is the activity equal to the fall produced by an intrafemoralvenous injection of 5×10^{-8} g of acetylcholine kg^{-1} in a urethane (1·8 g kg^{-1} i.p.)-anaesthetized rat of 250 g.

One unit corresponds to 0·045 μmol phosphate of the purified preparation. Each point represents the means ± standard errors of the means. Ordinate: decrease in carotid arterial blood pressure (mm Hg). Abscissa: dose of PD.D-I preparation (μmol phosphate kg^{-1}).

The depressor activity of PD.D-I was not affected significantly by incubation with proteolytic enzymes such as nagase and trypsin, it is therefore not an active peptide such as substance P or bradykinin. A fluorometric determination for PGE or PGF, based on an enzymic cycling system utilizing resazulin, NAD and PG dehydrogenase (Itami et al 1975), did not show the presence of either PGE or PGF in the purified preparation. PD.D-I tends to lose its activity on standing at room temperature and also in strong acid or alkali, i.e. after 8 h at 37 °C and pH 3·0 and 7·0, the recoveries of the hypotensive activity were about 80 and 100% respectively, and at pH 11·0 the recovery was only about 40% of the initial activity. The hypotensive activity of PD.D-I disappeared almost completely after treatment with phenylisocyanate or permanganate, and was decreased significantly by bromine. After treatment with triphenylphosphine or diazomethane in the cold (0 °C) the depressor activity was recovered without significant loss. The i.r. spectrum of PD.D-I indicates the general characteristics of phospholipids, and especially that of choline-containing phospholipids judging from a strong absorption at 970 cm^{-1}, however, there was no definite evidence of the presence of a ketonic group. The absorptions observed were at 3400, 2920, 2840, 1730, 1470, 1380, 1230, 1085 and 1050 cm^{-1}. In the n.m.r. spectrum PD.D-I showed the following signals (δ): 0·88, 1·3, 2·26, 3·30 and 3·6–4·6 ppm. The spectrum showed the general characteristics of saturated LPC but in the analysis of the PD.D-I preparation the molar ratio of glycerol (Renkonen 1962) or choline (Glick 1944; Argoudelis & Tobias 1975) to phosphorus was 0·99 and 0·97 respectively, whereas that of fatty acid methyl esters obtained by methanolysis and confirmed by g.c.-m.s. was 0·81. In the fatty acid composition the most predominant constituent was stearic acid (54·0%) followed by palmitic (39·4%), oleic (3·1%) and myristic acids (1·8%) in sequence; the content of other acids was negligible.

No methylals of long chain aldehydes were found in the preparation. Thus while the result of analysis of PD.D-I resembled LPC, the molar ratio of fatty acids to phosphorus was smaller than that of LPC. PD.D-I also resembled LPC in its behaviour during purification except for the properties cited above. Khairallah & Page (1960) reported that their hypertensive preparation from dog incubated plasma was possibly LPC, while synthetic stearoyl-LPC elicited a hypotensive response.

They considered that the reverse vaso-activities of LPCs might be due to variation of the fatty acid

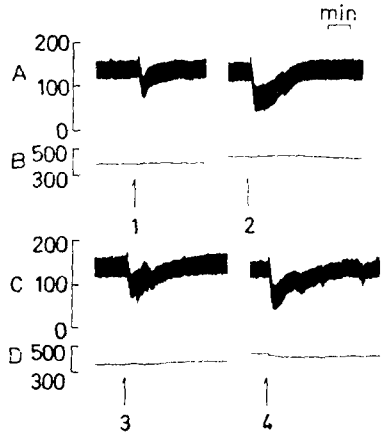

FIG. 2. Comparison of depressor effects between the purified preparation of PD.D-I and a mixed preparation of synthetic L-α-LPCs. The upper records show the respective blood pressure (A: mm Hg) responses and the heart rates (B: beats min^{-1}) of a pentobarbitone anaesthized rat (40 mg kg^{-1} i.p.) to the intravenous injections of 4 U kg^{-1} [0·18 μmol phosphate kg^{-1}, PD.D-I (1)] and 12 U kg^{-1} [0·54 μmol phosphate kg^{-1}, PD.D-I (2)] of the PD.D-I preparation. The lower records (C: blood pressure mm Hg; D: heart rate beats min^{-1}) show the responses to 4·52 μmol phosphate kg^{-1} [LPC (3)] and 13·55 μmol phosphate kg^{-1} [LPC (4)] of the mixed preparation of synthetic L-α-LPCs.

moiety or to the spatial arrangements of the LPCs. However, all synthetic L-α-LPCs we have tested exhibited depressor effects in anaesthetized rats (Tsukatani et al 1979) and their potencies were much lower than the PD.D-I preparation which showed a 25 fold higher hypotensive effect on the basis of phosphorus content than a mixture of synthetic L-LPCs prepared in parallel with the proportion of fatty acid composition of PD.D-I (Fig. 2). When PD.D-I preparation might be LPC consisted of common fatty acids, it is difficult to account for the intense depressor response, instability to alkali, positive reaction with 2,4-dinitrophenylhydrazine reagent on t.l.c., or retardation in mobility of a portion of the preparation on development with a solvent containing sodium bisulphite. Furthermore the gap in molar ratio between phosphorus and fatty acids seems to suggest a specific component resembling a fatty acid which is labile, convertible to a ketonic compound and responsible for potent hypotension. Recently, we reported the significant hypertensive action of natural and synthetic L-α-lysophosphatidic acid on anaesthetized rats and a hypotensive action on anaesthetized cats (Tokumura et al 1978a,b,c). Thus the lysotype of conformation seems to be a factor producing cardiovascular effects. PGs were kindly donated by Ono Pharmaceutical Industries, Ltd (Japan).

March 12, 1979

REFERENCES

Antonello, A., Baggio, B., Favaro, S., Corsini, A., Todesco, S., Borsatti, A. (1973) Pflügers Arch 341: 113–120

Argoudelis, C. A., Tobias, J. (1975) Anal. Biochem. 64: 276–280

Chalvardjian, A., Rudnicki, E. (1970) Ibid. 36: 225–226

Folch, J., Lees, M., Sistanley, G. H. (1957) J. Biol. Chem. 226: 497–509

Glick, D. (1944) Ibid. 156: 643–651

Itami, T., Fukuzawa, K., Takahashi, M., Tsukatani, H. (1975) Chem. Pharm. Bull. (Tokyo) 23: 1152–1155

Khairallah, P. A., Page, I. H. (1960) Am. J. Physiol. 199: 341–345

Martini, E., Chiesa, F., Oberosler, R., Maffeo, G., Secchi, C. (1967) Enzymologia 33: 100–112

Renkonen, C. (1962) Biochim. Biophys. Acta 56: 367–369

Tokumura, A., Akamatsu, Y., Yamada, S., Tsukatani H. (1978a) Agric. Biol. Chem. (Tokyo) 42: 515–521

Tokumura, A., Fukuzawa, K., Akamatsu, Y., Yamada, S., Suzuki, T., Tsukatani, H. (1978b) Lipids 13: 468–472

Tokumura, A., Fukuzawa, K., Tsukatani, H. (1978c) Ibid 13: 572–574

Tsukatani, H., Yamada, S., Tokumura, A., Miyamoto, T., Takauchi, K. (1976) Chem. Pharm. Bull. (Tokyo) 24: 2294–2300

Tsukatani, H., Yamada, S., Fukuzawa, K., Hamaguchi, C. (1979) J. Pharm. Pharmacol. 31: 110–111

Turcotte, J. G., Boyd, R. E., Quinn, J. G., Smeby, R. R. (1973) J. Med. Chem. 16: 166–168

Turcotte, J. G., Yu, C. S., Lee, H. L., Pavanaram, K. S., Sen, S., Smeby, R. R. (1975) Ibid. 18: 1184–1190