J. Pharm. Pharmacol. 1986, 38: 878–882
Received April 11, 1986

A platelet-aggregating and hypotensive phospholipid isolated from bovine brain

JUN-ICHI YOSHIDA, AKIRA TOKUMURA*, KENZI FUKUZAWA*, MOTONORI TERAO*, KENKICHI TAKAUCHI*, HIROAKI TSUKATANI*†

*School of Medicine, Tokushima University, Kuramoto-cho, Tokushima 770 and *Faculty of Pharmaceutical Sciences, Tokushima University, Sho-machi, Tokushima 770, Japan*

A phospholipid that differs from known active lipids and causes potent platelet aggregation and weak hypotension has been isolated from bovine brain. Its platelet aggregating effect on heparinized platelet-rich plasma from rabbits, was at a threshold concentration of about 0·2 nmol ml^{-1} as phosphorus. The effect was inhibited by CV-3988. The phospholipid was converted by diazomethane treatment to another active lipid that caused short-term hypotension, but not platelet aggregation, rather it inhibited the aggregation of rabbit heparinized platelets induced by platelet-activating factor.

Platelet-activating factor (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine, AGEPC, Paf-acether) has various potent biological effects besides platelet activation (Benveniste et al 1979; Demopoulos et al 1979). An antihypertensive, polar, renomedullary lipid obtained from an extract of the renomedulla by acetylation was also shown to be AGEPC (Bland et al 1979). A phospholipid preparation from the lipid fraction of bovine brain caused hypotension and was named Depressor-I (D-I) by Tsukatani et al (1976, 1978a, b, 1979). Though it showed similar biological activity to AGEPC, its chemical structure is not known. We report the separation of the preparation into two sub-fractions by further purification. One sub-fraction (D-I B) strongly aggregates platelets in platelet-rich plasma (PRP) and causes weak hypotension. This fraction, on diazomethane treatment, is converted to another active phospholipid (D-I B Met.) that has a strong short-term hypotensive effect, but no platelet-aggregating activity.

MATERIALS AND METHODS

Materials

CV-3988 (*rac*-3-(*N*-n-octadecylcarbamoyloxy)-2-methoxypropyl-2- thiazolioethyl phosphate) (Terashita et al 1983) were gifts from Takeda Chemical Industries Co. Ltd. AGEPC (hexadecyl), lyso-GEPC (1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine), lecithin (dipalmitoyl, PC), lysolecithin (palmitoyl, LPC), sphingomyelin (from bovine brain, SPM), lysophosphatidic acid (palmitoyl, LPA), ADP, arachidonic acid, indomethacin, phospholipase A_1, A_2, C and D were obtained from Sigma.

† Correspondence.

Preparation of biological active phospholipids

The partially purified hypotensive preparation from bovine brain (D-I) was obtained as reported by Tsukatani et al (1976). Briefly, the total lipid fraction was purified by fractionation with several solvents, and chromatography on two silicic acid and two Sephadex LH-20 columns. The active fraction was separated into two sub-fractions on a cellulose column. For this, cellulose (Toyo-Roshi, Type B, Japan) was dried over P_2O_5 for a week in a dessicator under vacuum. The 15 g of dried cellulose was suspended in methanol, poured into a glass column and washed with chloroform. The partially purified active preparation of D-I was dissolved in 3 ml of chloroform, applied to the column and allowed to stand overnight. The column was developed successively with 200 ml each of chloroform, chloroform–methanol mixtures of increasing polarity and finally methanol. Organic phosphorus in the eluates was determined according to Tsukatani et al (1978a). Preparative TLC was on silica gel plates (Merck TLC plates silica gel 60, 0·25 mm thickness) with acetone–methanol–water (1 : 2 : 1, v/v) as solvent. AGEPC, lyso-GEPC, LPC, PC and SPM were used as standards.

Diazomethane treatment

The hypotensive and platelet-aggregating activities of the eluates were measured before and after diazomethane treatment. For diazomethane treatment, a sample of eluate was dried, redissolved in 0·1 ml of chloroform and 1 ml of diazomethane ethereal solution added (de Boer & Backer 1954). The mixture was allowed to stand in a sealed tube at 4 °C for 24 h with occasional shaking, then dried.

Measurement of blood pressure

Male Wistar rats, 200 to 250 g, anaesthetized with pentobarbitone (40 mg kg^{-1} i.p.) were used for the assessment of the hypotensive effects of the samples. Basal mean blood pressure was maintained at 110 to 130 mmHg under anaesthetic. Arterial blood pressure was recorded through a cannula in the left carotid artery, with a pressure transducer (Nihon Kohden LPU-0·5) coupled to a multipurpose polygraph (Nihon Kohden RM-45). Test materials dissolved in 0·9% NaCl (saline) 0·2 to 0·4 ml were injected into the right jugular vein through a cannula which was flushed (saline) with 0·05 to 0·15 ml immediately after injection of the samples.

Aggregation of platelets

Rabbit blood was collected from the central ear artery of adult New Zealand white rabbits into 0·1 volume of heparin (20 iu ml^{-1} saline) or 3·8% trisodium citrate. Platelet-rich plasma (PRP) (approximately 3×10^8 platelets ml^{-1}) was obtained by centrifuging the blood at 100 g for 25 min at 20 °C. Platelet aggregation was monitored by continuously recording light transmission in a platelet aggregometer (HUSM-System Platelet Aggregometer, Rika Denki, Japan). Dried eluate samples were dissolved in saline containing 0·1% bovine serum albumin (BSA) to give 0·7–21 ng ml^{-1} phosphorus and 10 µl added to 250 µl of PRP. Arachidonic acid was dissolved in 0·1 M Na_2CO_3. Indomethacin was dissolved in water–ethanol (9:1, v/v) and then diluted with saline for tests.

RESULTS

On cellulose column chromatography, the partially purified platelet-aggregating and hypotensive preparation obtained from bovine brain (D-I), was separated into two sub-fractions as shown in Fig. 1. One sub-fraction (D-I B), which was mainly eluted from the column with the chloroform–methanol mixture (90 : 10 v/v) had a marked platelet activating effect on PRP but a weak hypotensive effect. This active fraction differed in its elution characteristic from AGEPC and the 1-acyl analogue of AGEPC, both of which were mainly eluted in chloroform–methanol mixture (95:5, v/v). D-I B was further purified by rechromatography on a cellulose or silicic acid column and reverse phase preparative TLC on silica gel plates with acetone–methanol–water (1:2:1, v/v) as solvent. The almost purified preparation of the platelet-aggregating and hypotensive phospholid, D-I B, was detectable with iodine vapour, ethanolic sulphuric acid or modified Dittmer reagent. The R_F values of D-I B resembled those of AGEPC, the 1-acyl analogue of AGEPC, LPC and

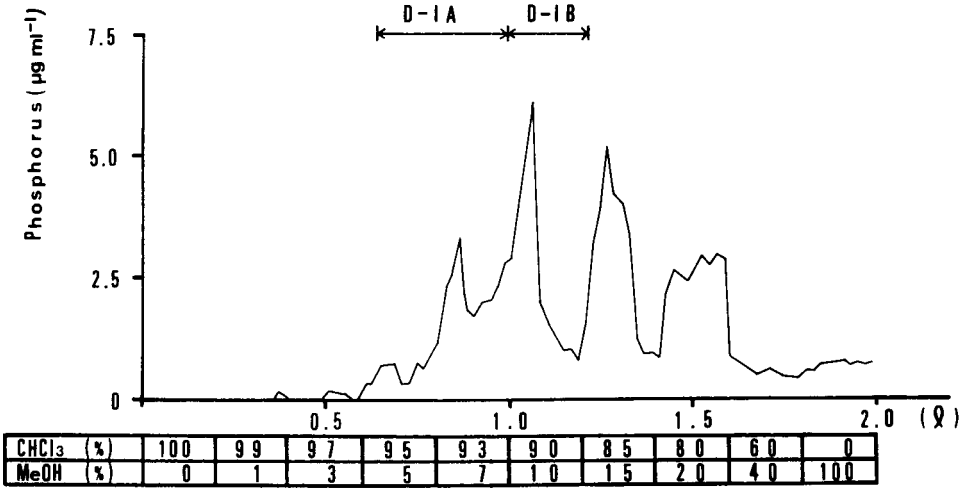

FIG. 1. Elution profile of D-I on a cellulose column. Partially purified active factor D-I (corresponding to 10 kg of bovine brain) was dissolved in 3 ml of chloroform, and applied to a cellulose (15 g) column. The column was developed with 200 ml each of chloroform, chloroform–methanol mixtures (99:1, 97:3, 93:5, 93:7, 90:10, 85:15, 80:20, 60:40, v/v) and finally methanol. Each 10 ml of the eluate from the column was collected, and organophosphorus was determined. The hypotensive effect on sodium pentobarbitone-anaesthetized rats (40 mg kg^{-1} i.p.) and platelet-aggregating effect on heparinized rabbit PRP in the eluates were also measured with and without diazomethane treatment. The eluate from the chloroform–methanol mixture (90:10, v/v) that causes weak hypotension and potent platelet-aggregation (D-I B) could be converted by diazomethane treatment to another active substance (D-I B Met.) that caused intense short-term hypotension but not platelet aggregation.

lyso-GEPC, but differed from those of PC, SPM and LPA (Table 1). D-I B was readily soluble in polar solvents and could be stored in a refrigerator as a syrup for six months. D-I B completely lost both its platelet-aggregating and hypotensive activities on treatment with phospholipase A_1, A_2, C or D.

Table 1. R_F values of D-I B.

Solvent	D-I B	AGEPC	LPC	PC	SPM	LPA
$CHCl_3$–MeOH–H_2O (65:35:5, v/v)	0·18	0·20	0·13	0·38	0·22	0·40
$CHCl_3$–MeOH–25%NH_4OH (65:35:4, v/v)	0·14	0·15	0·10	0·30	0·18	Or.
$CHCl_3$–MeOH–AcOH–H_2O (25:15:4:2, v/v)	0·24	0·28	0·17	0·55	0·31	0·29

Merck TLC plate silica gel 60 (0·25 mm thickness); $CHCl_3$, chloroform; MeOH, methanol; AcOH, CH_3COOH; Or., origin. Visualized with iodine vapour.

The hypotensive effect of D-I B on the anaesthetized rat was much weaker than that of AGEPC, whereas the D-I B Met. had a strong short-term hypotensive effect when injected i.v. at 7 nmol kg^{-1} as phosphorus (corresponding to 2·2 g of wet weight of nervous tissue kg^{-1}) as shown in Fig. 2. After treatment of the rat with CV-3988, a typical inhibitor of AGEPC, the response to D-I B, like the response to AGEPC was markedly inhibited, but the response to D-I B Met. was not affected.

The typical platelet aggregation activity of D-I B at various concentrations on rabbit heparinized PRP is shown in Fig. 3A. In general, D-I B at a low concentration (0·7 nmol ml^{-1} as phosphorus) caused reversible aggregation of platelets to a similar extent in heparinized and citrated rabbit PRP. With D-I B concentrations of up to about 0·7 nmol ml^{-1} as phosphorus, aggregation increased in a dose-related manner and was reversed after 1–2 min. The threshold dose of D-I B was approximately 0·2 nmol ml^{-1} as phosphorus. Higher concentrations of D-I B induced irreversible aggregation of platelets in both heparinized and citrated PRP preparations. The platelet-aggregating potency of D-I B was stronger than that of the 1-acyl analogue of AGEPC, but much weaker than that of AGEPC, when the comparison is made on the basis of phosphorus content. CV-3988, an inhibitor of AGEPC, inhibited platelet aggregation by D-I B (Fig. 3B). Because indomethacin, 50 μM, inhibited the irreversible aggregation of rabbit platelets by arachidonic acid, but not by D-I B at any concentration tested, the effect of D-I B on platelet aggregation was not due to endogenous prostaglandin synthesis. Rabbit heparinized PRP exposed to that concentration of D-I B inducing reversible aggregation was less sensitive on subsequent treatment (Fig. 3C).

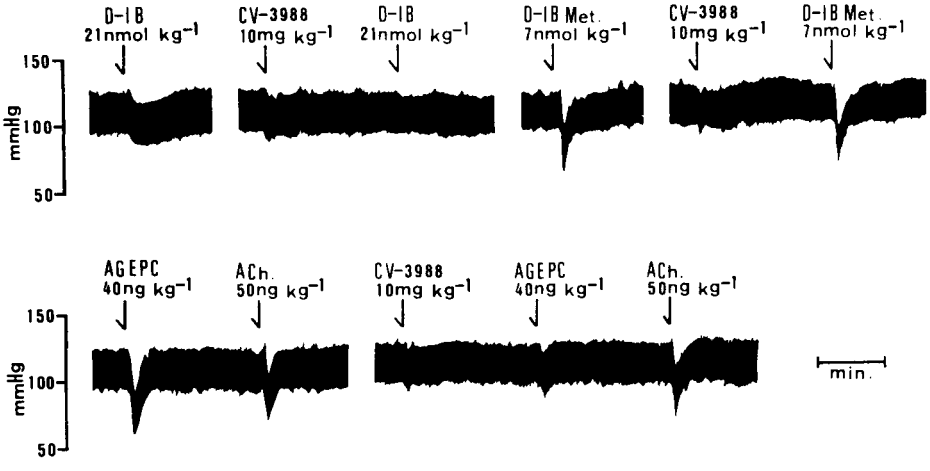

FIG. 2. Blood pressure responses of a male Wistar rat, 250g, anaesthetized with sodium pentobarbitone (40 mg kg^{-1} i.p.) to D-I B (21 nmol kg^{-1} as phosphorus corresponding to 6·5 g of wet weight of nervous tissue kg^{-1}), D-I B Met. (7 nmol kg^{-1} as phosphorus corresponding to 2·2 g wet weight of nervous tissue kg^{-1}), AGEPC (40 ng kg^{-1}) and acetylcholine (ACh, 50 ng kg^{-1}), and the effects of CV-3988 (10 mg kg^{-1}) on these responses. Samples were given intravenously at the points indicated by arrows. The hypotensive effect on D-I B Met. was not inhibited by pretreatment of the rat with CV-3988. Data are typical results of five separate experiments.

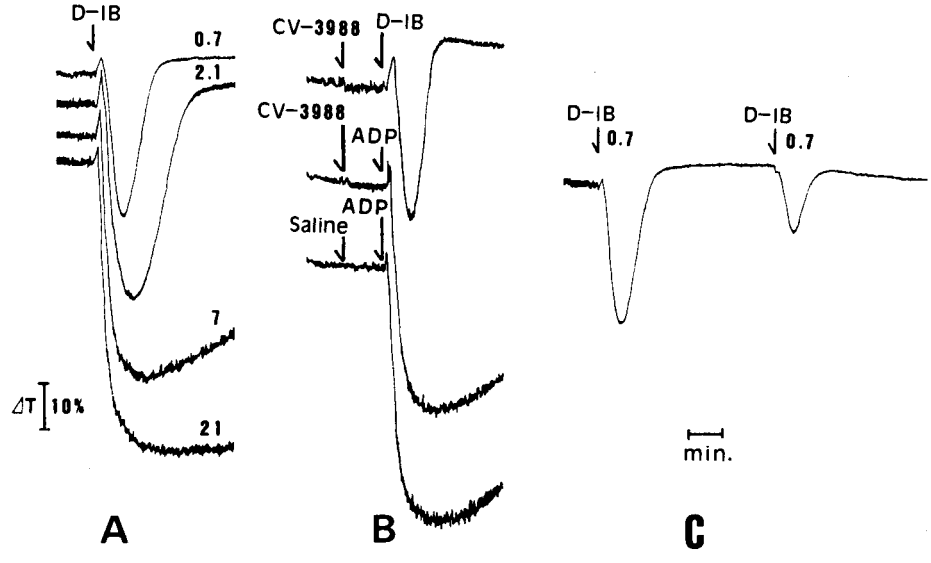

FIG. 3. A, Platelet-aggregating effect of D-I B on heparinized rabbit PRP at doses of 0·7, 2·1, 7 and 21 nmol ml^{-1} as phosphorus. The dose of 0·7 nmol ml^{-1} corresponds to 0·22 g of wet weight of nervous tissue ml^{-1}. B, Inhibitory effects of CV-3988 (3×10^{-5} M) on platelet-aggregating activities of D-I B (21 nmol ml^{-1} as phosphorus) and ADP (1×10^{-5} M). C, Tachyphylaxis of heparinized rabbit PRP treated with D-I B (0·7 nmol ml^{-1} as phosphorus). ΔT = change in light transmission. Data are typical results of seven separate experiments.

Treatment of D-I B with diazomethane to give D-I B Met. resulted in complete loss of platelet-aggregating activity, only its modifying effect on the shape of platelets remaining (Fig. 4A). On the other hand, D-I B Met. showed a potent short-term hypotensive effect in anaesthetized rats (Fig. 2). The shape modification of the platelets increased with increase in the concentration of D-I B Met. Furthermore, D-I B Met. inhibited the platelet-aggregating activity of AGEPC (Fig. 4B). That is, when platelets had been pretreated with a low concentration of D-I B Met. and then allowed to return to the normal state, the platelet aggregating activity of added AGEPC was inhibited, and the inhibition was dose-dependent.

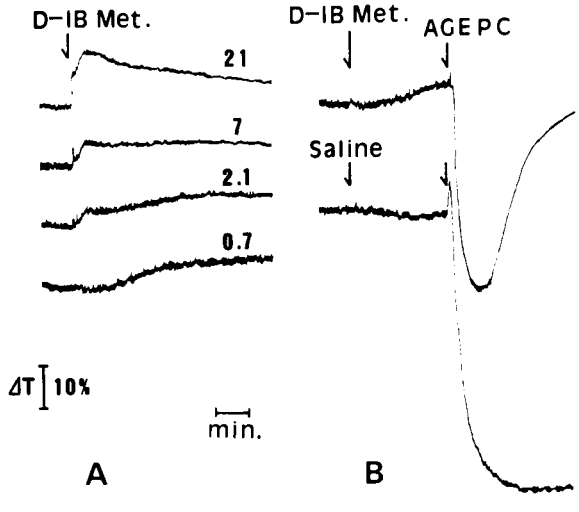

FIG. 4. A, Shape-modification of heparinized rabbit platelets by D-I B Met. The platelet aggregating effect of D-I B disappeared on conversion to D-I B Met. with diazomethane. B, Inhibitory effect of D-I B Met. (0·7 nmol ml^{-1} as phosphorus) to the rabbit platelet aggregation induced by AGEPC (3×10^{-8} M). ΔT = change in light transmission. Data are typical results of seven separate experiments.

DISCUSSION

As described above, D-I B, a hypotensive and platelet-aggregating phospholipid obtained from bovine nervous tissue in a nearly pure state, showed a similar effect to AGEPC. But it was fundamentally different in behaviour after treatment with diazomethane or phospholipase A_1. After treatment of AGEPC with diazomethane, the resulting preparation had less biological activity, but, AGEPC was unaffected by phospholipase A_1 pretreatment. AGEPC is known to have various biological activity, such as hypotensive, smooth muscle-stimulating, polymorphonuclear leucocyte-activating, anti-

spasmodic and other effects, besides a platelet-activating effect. These effects make AGEPC unsuitable for therapy. In the present investigation, we obtained a substance (D-I B Met.) by diazomethane treatment of a phospholipid from bovine brain. This has potent depressor activity but no platelet aggregating activity. It therefore might be useful as a basis for a new type of drug having hypotensive activity similar to AGEPC but a different mechanism from known drugs, and without the platelet-aggregating activity.

REFERENCES

Benveniste, J., Tencé, M., Varenne, P., Bindault, J., Boullet, C., Polonsky, J. (1979) C. R. Acad. Sci. Paris 289 (sér. D.) 1037–1040

Bland, M. L., Snyder, F., Byers, L. W., Brooks, B., Muirhead, E. E. (1979) Biochem. Biophys. Res. Commun. 90: 1194–1200

de Boer, T. J., Backer, H. J. (1954) Res. Trav. Chim. Pays-Bas 73: 229–234

Demopoulos, C. A., Pinckard, R. N., Hanahan, D. J. (1979) J. Biol. Chem. 254: 9355–9358

Terashita, Z., Tsushima, S., Yoshioka, Y., Nomura, H., Inada, Y., Nishikawa, K. (1983) Life Sci. 32: 1975–1982

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

Tsukatani, H., Yamada, S., Fujii, M., Awaji, T., Okamoto, M., Itami, T. (1978a) Ibid. 26: 3271–3280

Tsukatani, H., Yamada, S., Takauchi, K., Tokumura, A., Tatsumichi, H., Kumegawa, K. (1978b) Ibid. 25: 3281–3288

Tsukatani, H., Yamada, S., Tokumura, A., Itami, T. (1979) Jap. J. Pharmacol. 29: 695–705