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TRIPHENYLTIN FLUORIDE *IN VITRO* INHIBITION OF RABBIT PLATELET COLLAGEN-INDUCED AGGREGATION AND ATP SECRETION AND BLOCKADE OF ARACHIDONIC ACID MOBILIZATION FROM MEMBRANE PHOSPHOLIPIDS

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Abstract—Recent studies have demonstrated that triphenyltin fluoride (TPTF) inhibits collagen-induced aggregation and ATP secretion of rabbit platelets *in vivo* [S. Manabe and O. Wada, *J. Toxic. Sci.* **6**, 236 (1981)].

The aim of the present investigation was to test the effects *in vitro* of TPTF on platelet aggregation and to elucidate the mechanism of the inhibitory action by studying the release and metabolism of arachidonic acid and the cyclic AMP contents of rabbit platelets treated *in vitro* with TPTF. Although no inhibitory effect of TPTF was found on sodium arachidonate-induced platelet aggregation and ATP secretion, TPTF inhibited both reactions induced by collagen. Triphenylarsine and triphenylantimony did not inhibit, even at a concentration of 10^{-3} M. The anti-aggregating concentration (IC_{50}) of TPTF was 6.0×10^{-6} M against collagen. TPTF had no inhibitory effect on the conversion of exogenous arachidonic acid to malondialdehyde (MDA) by platelets, while the collagen-induced production of arachidonate metabolites [MDA, 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and thromboxane B_2] was remarkably inhibited by TPTF. Furthermore, TPTF apparently inhibited the collagen-induced release of arachidonic acid from platelets, although the formation of phosphatidic acid was not inhibited. Total cyclic AMP content after TPTF exposure was not changed significantly. These results indicate that TPTF inhibited the collagen-induced arachidonic acid release from platelet phospholipids, presumably by acting on phospholipase A_2. Furthermore, it seems unlikely that the inhibition of arachidonic acid release by TPTF can be explained by the level of cyclic AMP in platelets.

Organotin compounds, which are widely used as agricultural chemicals and marine antifoulants [1], have been studied intensively from the stand-point of toxicity. However, no attention has been paid to the effect of organotin compounds on platelet function [2–4]. Furthermore, the effects of organotins on prostaglandin metabolism and on the stimulus-reaction system of membrane have not been reported in recent studies.

In the course of the toxicological study of TPTF*, we found that rabbits and rats treated with this compound showed nasal and conjunctive bleeding. Hematological examination revealed a prolongation of both clotting time and bleeding time without any abnormalities in platelet counts and partial thromboplastin time. Because these findings suggested a disturbance in platelet function, we turned our attention to platelets and found that platelets obtained from rabbits treated with TPTF exhibited inhibition of collagen-induced aggregation and ATP secretion [5]. In the present investigation, we ascertained *in vitro* that a low concentration of TPTF also inhibited collagen-induced aggregation and ATP secretion of platelets from normal rabbits. In addition, to elucidate the mechanism of TPTF inhibition, we investigated arachidonate metabolism as well as cyclic AMP contents, using platelets treated *in vitro* with TPTF. Furthermore, after comparing the effects of other triphenyl metals and aspirin with those of TPTF on platelet-aggregation responses, we have concluded that TPTF has specific inhibitory effects on platelet-aggregation and ATP secretion by inhibiting the release of arachidonic acid from the membrane.

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* Abbreviations: TPTF, triphenyltin fluoride; MDA, malondialdehyde; HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; and TX, thromboxane.

MATERIALS AND METHODS

Blood collection and preparation of plasma. Venous blood was collected from male Japan White rabbits (2.2 to 2.5 kg body weight) that had been starved for 12 hr. Administration of drugs and chemical agents which might affect platelet function was carefully avoided for a month. Blood (9 vol.) was collected in 1 vol. of 3.8% trisodium citrate as an anticoagulant.

The citrated blood was gently mixed and centrifuged at 180 *g* for 20 min at 20°, and the supernatant (citrated-platelet rich plasma; C-PRP) fraction was carefully transferred to a plastic tube by plastic

pipette. The platelet concentration of C-PRP was adjusted to approximately 300,000/μl by addition of an appropriate amount of citrated-platelet poor plasma (C-PPP) prepared by centrifugation of the remainder of the blood at 3000 *g* for 20 min at 4°. Platelet concentration in C-PRP was measured with a platelet counter (Toua model PL-100, Tokyo, Japan).

Preparation of solutions of triphenyl metals. TPTF, triphenylarsine and triphenylantimony were obtained from the Nitto Kasei Co., Ltd. (Osaka, Japan). *N,N*-Dimethylformamide was obtained from the Wako Co., Ltd. (Osaka, Japan). The stock solutions of triphenylarsine, triphenylantimony and aspirin (Shionogi, Osaka), containing 1 to 10^{-3} moles/l, were prepared by dissolving them in dimethylformamide. For TPTF, only solutions containing 10^{-2} to 10^{-3} moles/l were prepared, because of the poor solubility of the compound in dimethylformamide. Each solution of the triphenyl metals was diluted 100-fold with saline just before aggregation measurements.

Aggregation and ATP secretion measurements. Platelet aggregation and ATP secretion were recorded at 37° and 1.0×10^3 rpm according to the method of Detwiler and Feinman [6] in a Lumi-Aggregometer (Chrono-log Co., Havertown, PA). The Lumi-Aggregometer is an instrument for simultaneous measurement of secretion and aggregation by platelets [7]. The extent of aggregation was measured as percent light transmission, using light transmission of C-PRP as 0% and that of C-PPP as 100%.

The secretion of ATP that occurred during the release reaction was measured by detecting the luminescence in the luciferase–luciferin system [6]. Aggregating substances formed 1/10 of the final sample volume in the aggregater. Collagen reagent (HORM) was obtained from Hormon-Chemie (München, West Germany) and diluted in HORM buffer. Sodium arachidonate and arachidonic acid (Sigma) were prepared and kept under nitrogen as described by Silver *et al.* [8].

The luciferase–liciferin reagent (CHRONO-LUME, Chrono-log) was prepared by dissolving 100% of the vial contents in 5 ml of sterile water to give a final concentration of 40 mg/ml.

A 0.1-ml sample of the solution containing one of the triphenyl metals, aspirin or dimethylformamide (1% in saline) was added to 0.9 ml C-PRP and preincubated at 37°. Aggregation and ATP secretion measurements were performed using 400 μl of the luciferase–luciferin reagent solution.

In preliminary experiments, measurements of ATP by detection of the luminescence of the luciferase–luciferin system were not disturbed by TPTF, even at a final concentration of 10^{-5} M.

Chromatographic separation of phosphatidic acid, arachidonic acid and its metabolites. Samples of 50 ml of C-PRP were incubated at 37° for 2 hr with 2 μCi of [^{14}C]arachidonic acid (Amersham Corp.). At the end of the incubation period, EDTA was added to give a final concentration of 2 mM, and the platelets were pelleted by centrifugation at 3000 *g* for 20 min at 4°. The pellet was resuspended gently in 50 ml NaCl/Tris–HCl (120 mM/30 mM) buffer, pH 7.4 (Tris/NaCl buffer).

One-milliliter samples of the labeled platelets (1×10^9 cells/ml) were preincubated with either 0.1% dimethylformamide (control) or 10^{-5} M triphenyltin fluoride for 15 min at 37°. Collagen was added to give a final concentration of 20 μg/ml, and the mixture was incubated at 37° for different periods of time. Lipid extraction of the whole incubation mixture was done according to Billah *et al.* [9]. Dried

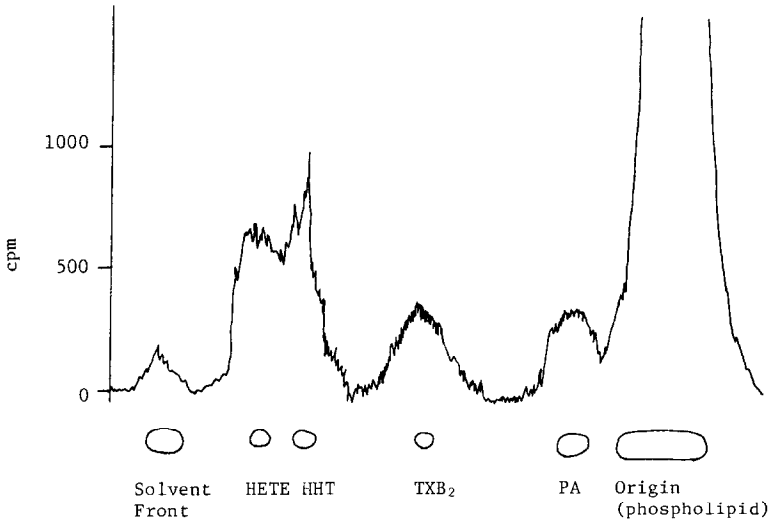

Fig. 1. Separation of radioactive arachidonate metabolites by thin-layer chromatography. Platelets were prelabeled with [^{14}C]arachidonic acid and then exposed to collagen (20 μg/ml) and extracted. The plates were run with a top phase of ethylacetate–2,2,4-trimethylpentane–acetic acid–water (90:50:20:100, by vol.). The result, obtained with a chromatogram scanner after visualization by iodine staining, is shown. Abbreviations: PA, phosphatidic acid; TXB_2, thromboxane B_2; HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; and HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid.

lipids were dissolved in $CHCl_3$ and spotted on silica gel G plates (Merck).

The solvent used for separation was the upper phase from a mixture of ethylacetate–2,2,4-trimethylpentane–acetic acid–water (90:50:20:100, by vol.), which give a good separation of cyclooxygenase and lipoxygenase products and of phosphatidic acid [10]. In this system, arachidonic acid migrates between HETE and the solvent front, and triglyceride moves with the solvent front. The R_f value for arachidonic acid was 0.87. All the other labeled phospholipids remained at the origin (Fig. 1). The spots were visualized by iodine staining, and the radioactivity was determined by liquid scintillation counting. Standard HETE, HHT and TXB_2 were made available by members of the Experimental Chemistry Unit of The Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Phosphatidic acid was obtained from the Green Gross Co., Ltd. (Osaka, Japan).

Collagen-induced release of radioactive arachidonic acid from [^{14}C]arachidonic acid-labeled platelets was determined by addition of phenidone (Tokyo Kasei Co., Ltd, Tokyo, Japan). Phenidone was added to the reaction mixture to give a final concentration of 0.25 mM before collagen stimulation [11].

Measurements of MDA formation induced by sodium arachidonate and collagen. MDA production was measured by the spectrometric assay of Smith *et al.* [12] as modified by Villa *et al.* [13]. Citrated-platelet rich plasma (C-PRP) was cooled to 4°, and EDTA was added to give a final concentration of 1 mM. The C-PRP was centrifuged at 2000 *g* for 20 min at 4°. The supernatant fraction was discarded, and the platelet pellet was resuspended in an equal volume of buffered saline containing EDTA (1 mM EDTA, 0.15 M phosphate buffer, pH 7.4). The platelets were centrifuged at 2000 *g* for 15 min at 4° and finally resuspended in an appropriate volume of the above solution without EDTA. A 0.9-ml sample of the platelet suspension, containing 6.0×10^5 platelets/μl, was preincubated with 0.1 ml of either dimethylformamide (1%), aspirin or TPTF solution at 37° for 15 min before addition of the stimulating agent. The reaction was stopped by adding 0.5 ml of 100% trichloroacetic acid after 5 min of incubation with the stimulating agent; this procedure removed all plasma proteins, and filbrin clots that had formed, in the samples with agents. All specimens were processed as described by Villa *et al.* [13]. Readings at 532 nm were taken with a Hitachi 124 dual-beam spectrometer, and the results were expressed as nmoles/1.0×10^9 platelets. Preliminary experiments showed a linear relation between the platelet count in C-PRP (150,000–1,500,000/μl) and the amount of MDA formed in our system by each stimulus. In addition, TPTF did not influence the measurements of MDA.

Determination of the cyclic AMP content of platelets. Cyclic AMP was measured with a protein binding technique (Amersham kit) [14]. EDTA was added to C-PRP to give a final concentration of 2 mM, and the platelets were pelleted by centrifugation at 3000 *g* for 20 min at 4°.

The pellet was resuspended gently in Tris/NaCl buffer, pH 7.4, and centrifuged once more at 3000 *g* for 20 min. The pellet was finally resuspended in Tris–buffer saline medium consisting of 25 mM Tris–HCl, 116 mM NaCl, 4.17 mM KCl, 1.8 mM KH_2PO_4, 1.18 mM $MgCl_2$, and 5 mM glucose, pH 7.4. Platelet number was adjusted to 1×10^9 cells/ml. Samples of a 4.5-ml platelet suspension were incubated with either 500 μl of 1.0% dimethylformamide or 500 μl of 10^{-4} M triphenyltin fluoride solution for 15 min at 37°.

Samples of 500 μl were taken at appropriate time intervals. Deproteinization was achieved by adding 100 μl of 50% trichloroacetic acid. After centrifugation, the protein-free supernatant fraction was extracted four times with water-saturated ether, after which the cyclic AMP content in the supernatant fraction was determined and expressed in pmoles/10^9 platelets. The recovery value of cyclic AMP in this procedure was 91.8 ± 3.0% (mean ± 1 S.E.M.).

Measurements of endogenous TXB_2 production induced by collagen. Platelets suspended in Tris/NaCl buffer, containing 6.0×10^5 platelets/μl, were preincubated with either 10^{-5} M TPTF or 0.1% dimethylformamide (control) for 15 min at 37°. After the preincubation, collagen was added to the platelet suspension to give a final concentration of 20 μg/ml, and the mixture was incubated at 37°.

Samples of 400 μl were taken at appropriate time intervals. TXB_2 was measured using an established radioimmunoassay [15]. In preliminary experiments, radioimmunoassay of TXB_2 was not disturbed by TPTF, even at a final concentration of 10^{-5} M.

Statistics. The data were analyzed by Student's *t*-test for significance of differences.

RESULTS

Effect of preincubation with TPTF on collagen-induced aggregation and ATP secretion of platelets. As shown in Fig. 2, the intensity of the inhibitory effects of TPTF (6.7×10^{-6} M) on collagen-induced aggregation and ATP secretion were parallel for preincubations of 1, 5, and 15 min; preincubation of 60 min with TPTF strikingly inhibited the collagen (20 μg/ml)-induced aggregation and ATP secretion. It should be noted that sodium arachidonate (0.25 mM) induced apparent aggregation and ATP secretion of the TPTF-treated platelets that showed no aggregation and ATP secretion within 20 min after addition of collagen (20 μg/ml).

Effect of triphenyl metals on platelet aggregation induced by collagen and sodium arachidonate. As can be seen in Table 1, TPTF inhibited collagen (20 μg/ml)-induced platelet aggregation at concentrations higher than 6.0×10^{-6} M. The (IC_{50}) of TPTF was 6.0×10^{-6} M against collagen-induced aggregation. In contrast, sodium arachidonate-induced platelet aggregation (Table 1) and ATP secretion (data not shown) were not inhibited significantly by TPTF. Aspirin at a concentration of 1.0×10^{-3} M remarkably inhibited platelet aggregation induced by both collagen and sodium arachidonate. The other triphenyl metals did not inhibit either collagen or sodium arachidonate-induced platelet aggregation at any of the concentrations, up to 10^{-3} M. In addi-

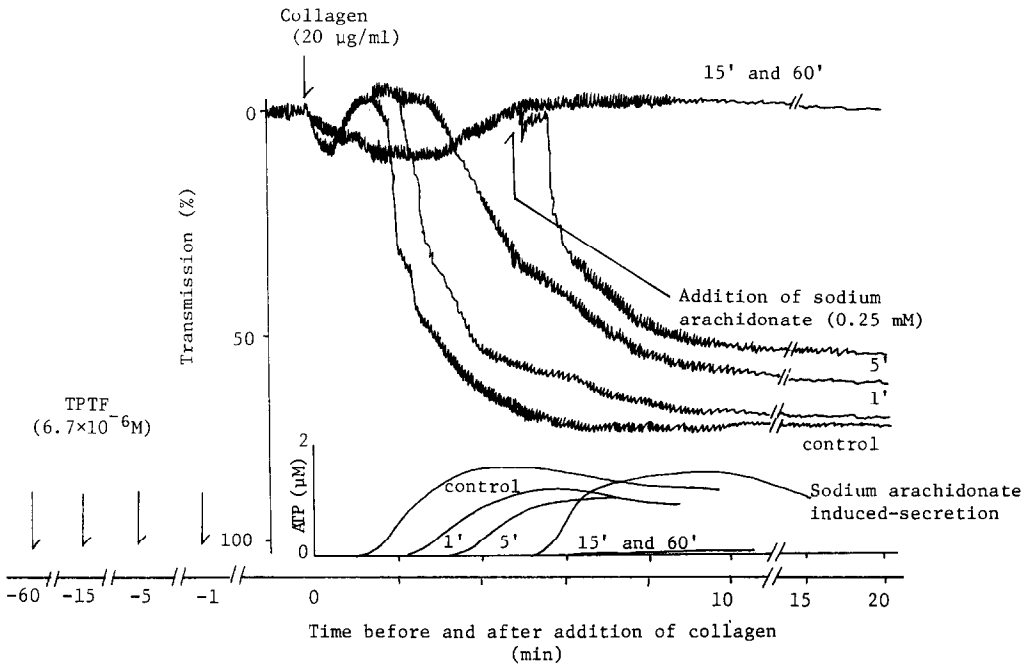

Fig. 2. Effects of preincubation of platelets with triphenyltin fluoride (TPTF) on collagen-aggregation (upper curves) and ATP secretion (lower curves). After normal platelets were preincubated with 6.7×10^{-6} M TPTF for 1, 5, 15 or 60 min, collagen-aggregation and ATP secretion were tested. Sodium arachidonate (0.25 mM) induced aggregation and ATP secretion of the TPTF-treated platelets that showed no aggregation or ATP secretion within 20 min of collagen addition. Control was preincubated with 0.1% dimethylformamide for 60 min. Results shown are typical of five experiments.

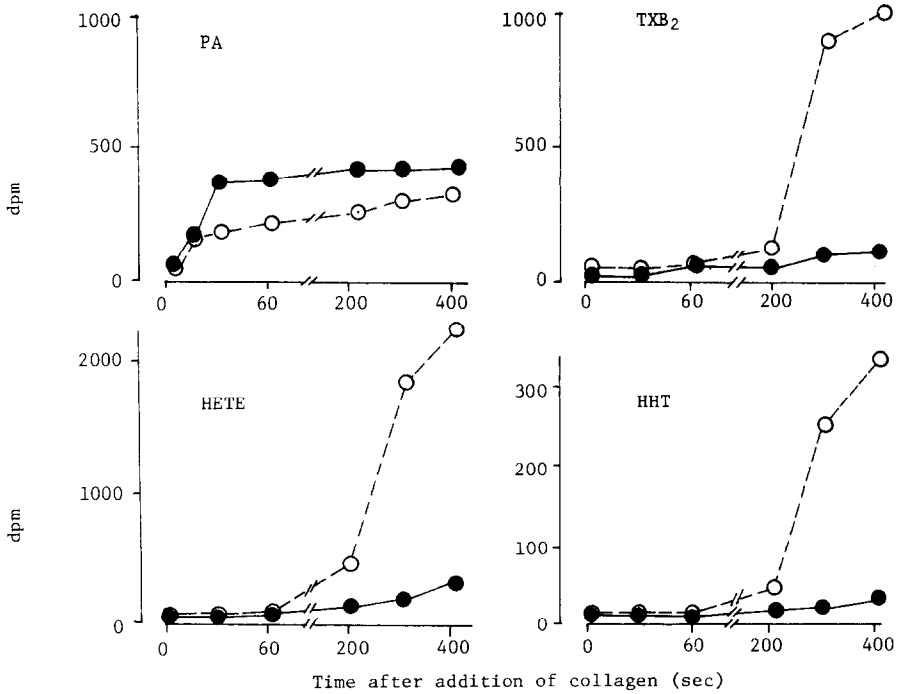

Fig. 3. Effects of triphenyltin fluoride (TPTF) on collagen-induced formation of lipoxygenase and cyclooxygenase products and of phosphatidic acid. [^{14}C]Arachidonate-labeled platelets (1.0×10^9/ml) were preincubated with either 10^{-5} M TPTF or 0.1% dimethylformamide (control) for 15 min, followed by addition of collagen. Each point represents the mean of two experiments. Key: (○) control values, and (●) results obtained from the TPTF-treated platelets. Abbreviations: PA, phosphatidic acid; TXB_2, thromboxane B_2; HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; and HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid.

Table 1. Effects of triphenyl metals on platelet aggregation induced by collagen (20 μg/ml) and sodium arachidonate (0.25 mM)*

	Collagen-induced aggregation (%)					Sodium arachidonate-induced aggregation (%)				
	Final concentration of triphenyl metals and aspirin					Final concentration of tryphenyl metals and aspirin				
	1.0×10^{-6} M	6.0×10^{-6} M	6.7×10^{-6} M	1.0×10^{-5} M	1.0×10^{-3} M	6.7×10^{-6} M	1.0×10^{-5} M	1.0×10^{-3} M		
Triphenyltin fluoride	74 ± 3.1	37 ± 4.5†	9.0 ± 2.3‡	5.3 ± 0.8‡		78 ± 3.0	75 ± 3.4			
Triphenylarsine	75 ± 2.0	73 ± 2.5	74 ± 2.0	73 ± 4.1	71 ± 3.5	77 ± 3.3	78 ± 2.1	77 ± 3.2		
Triphenyl antimony	74 ± 3.1	75 ± 3.3	74 ± 2.8	71 ± 3.2	71 ± 3.5	76 ± 3.7	77 ± 3.4	78 ± 3.6		
Aspirin	72 ± 2.5	73 ± 3.5	75 ± 2.0	68 ± 4.0	9.5 ± 2.0‡	77 ± 3.0	67 ± 4.9	9.6 ± 2.1‡		
Control			75 ± 2.5				78 ± 3.2			

* The extent of aggregation is expressed as percent light transmission. Values indicate the means ± 1 S.E.M. of five experiments performed in C-PRP preincubated for 15 min with triphenyl metals and aspirin. Controls were preincubated with 0.1% dimethylformamide solution.
† $P < 0.01$, as compared with control
‡ $P < 0.001$, as compared with control.

tion, inhibition of ATP secretion was not seen without inhibition of platelet aggregation.

Effect of TPTF on collagen-induced production of arachidonate metabolites and phosphatidic acid. The increases in the radioactive arachidonate metabolites (TXB_2, HHT and HETE) due to collagen stimulation were apparently inhibited by TPTF, although there was a slight increase in production of these cyclooxygenase and lipoxygenase compounds from the TPTF-treated platelets at 300 sec after addition of collagen (Fig. 3). However, collagen-induced production of phosphatidic acid from the TPTF-treated platelets was not inhibited.

Effect of TPTF on collagen-induced release of radioactive arachidonic acid from [^{14}C]arachidonic acid-labeled platelets. Phenidone (2.5 mM) was used in order to accumulate arachidonic acid released from platelet phospholipids by inhibiting both cyclooxygenase and lipoxygenase pathways in platelets [16]. Figure 4 shows the inhibitory effect of TPTF on the collagen-induced release of radioactive arachidonic acid from [^{14}C]arachidonic acid-labeled platelets. The release of radioactive arachidonic acid from platelet phospholipids in the control was 1.9 ± 0.2% (mean ± 1 S.E.M.). Although a slight increase in the release of arachidonic acid from the TPTF-treated platelets was observed at 300 sec after addition of collagen, arachidonic acid release was apparently inhibited by TPTF.

MDA formation of TPTF-treated platelets following stimulation by sodium arachidonate and collagen. Table 2 shows that, when sodium arachidonate was used to stimulate MDA formation, TPTF did not inhibit MDA formation, although collagen-induced MDA formation was almost completely inhibited in the TPTF-treated platelets. On the other hand, aspirin completely inhibited MDA formation induced by both arachidonate and collagen.

These results not only indicate that exogenous arachidonic acid was metabolized by the TPTF-treated platelets but also agree with the results that show that TPTF did not affect the arachidonate-induced aggregation and ATP secretion, in contrast to the complete inhibition of collagen-induced aggregation and ATP secretion.

Effect of TPTF on cyclic AMP content of platelets. The effect of TPTF on total cyclic AMP content of rabbit platelets was investigated, because there is evidence that elevation of platelet cyclic AMP is accompanied by inhibition of arachidonic acid release from platelet phospholipids. As shown in Table 3, incubation of platelets with TPTF did not affect the total cyclic AMP content of platelets, although dimethylformamide as control slightly though not significantly, elevated the cyclic AMP content.

Effect of TPTF on the production of endogenous TXB_2 induced by collagen. The effect of TPTF on the production of endogenous TXB_2 was investigated, because incorporation of exogenous arachidonic acid into membrane phospholipids pools may result in other than normal specific activities of arachidonic acid in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. Figure 5 shows the inhibitory effect of TPTF on the collagen-induced production of TXB_2 from platelets.

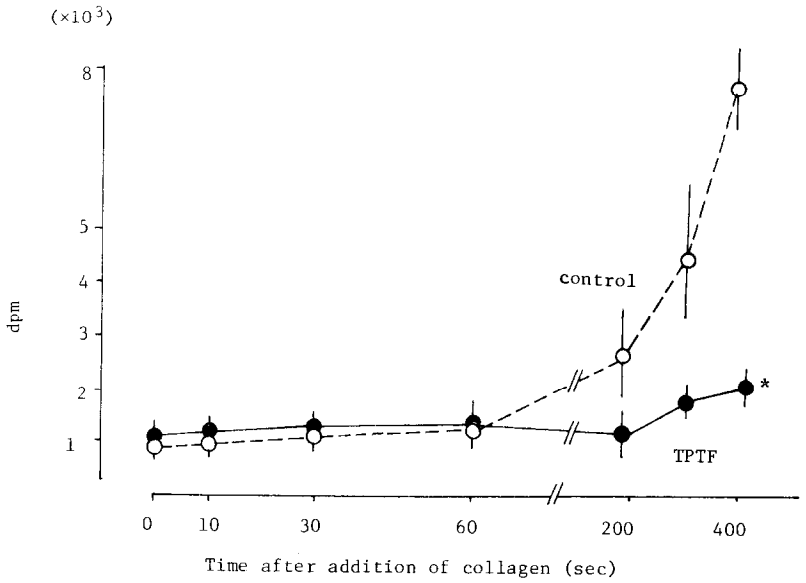

Fig. 4. Effect of triphenyltin fluoride (TPTF) on collagen-induced release of radioactive arachidonic acid from [^{14}C]arachidonic acid-labeled platelets. Preincubation with 10^{-5} M triphenyltin fluoride or 0.1% dimethylformamide (control) was carried out for 15 min at 37° followed by addition of collagen (20 μg/ml). Each point represents the mean ± 1 S.E.M. of three experiments. Key: (*) $P < 0.001$, as compared with control.

Table 2. Platelet malondialdehyde (MDA) formation induced by sodium arachidonate and collagen*

Stimulus	No. of experiments	(nmoles MDA formed/10^9 platelets) Dimethyl formamide	Triphenyltin fluoride (6.7×10^{-6} M)	Aspirin (1.0×10^{-3} M)
Collagen (20 μg/ml)	5	2.65 ± 0.20	0.10 ± 0.06†	0.12 ± 0.10†
Sodium arachidonate (0.25 mM)	5	7.35 ± 0.51	6.36 ± 0.25	0.12 ± 0.05†

* Values are means ± 1 S.E.M. of experiments performed in C-PRP preincubated for 15 min with either dimethylformamide (0.1%) as control, aspirin or triphenyltin fluoride.
† $P < 0.001$, as compared with control.

A collagen-induced production of TXB_2 in the control apparently occurred as early as 200 sec after addition of collagen. In contrast, TXB_2 was undetectable in the TPTF-treated platelets even at 600 sec after addition of collagen.

DISCUSSION

It is generally accepted that metabolites of arachidonic acid are important to platelet function. TXA_2, was well as prostaglandin G_2 and prostaglandin H_2, can induce platelet aggregation and the release of serotonin from storage granules [17]. Since there is little or no free arachidonic acid in the platelet, regulation of the production of these arachidonate

Table 3. Effect of triphenyltin fluoride on cyclic AMP content of platelets*

	(pmoles cyclic AMP/10^9 platelets) Incubation time (min) 0	5	15
Triphenyltin fluoride	31 ± 1.5	30 ± 2.0	30 ± 2.6
Dimethyl-formamide	30 ± 1.5	34 ± 2.4	36 ± 2.3

* Washed platelets were incubated with either 0.1% dimethylformamide (control) or 10^{-5} M triphenyltin fluoride at 37°. Values are the means ± S.E.M. of four experiments.

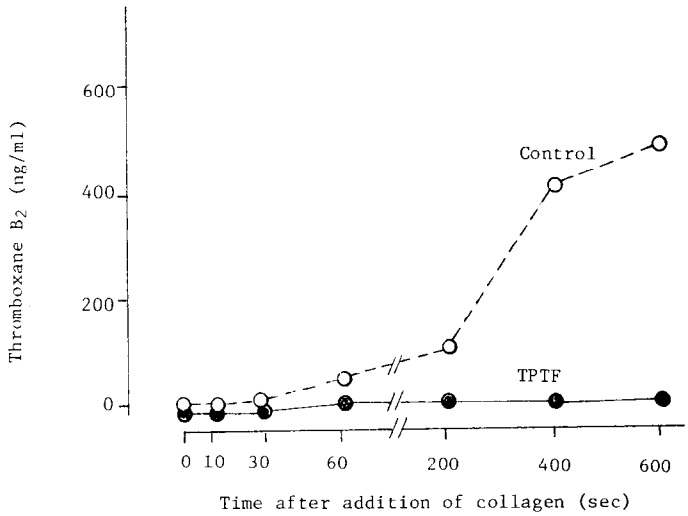

Fig. 5. Effect of triphenyltin fluoride (TPTF) on the production of endogenous TXB_2 induced by collagen. Platelets suspended in Tris/NaCl buffer were preincubated with either 10^{-5} M TPTF or 0.1% dimethylformamide (control) for 15 min, followed by addition of collagen (20 μg/ml). Samples were collected at the indicated intervals (sec, horizontal scale), and TXB_2 was determined by radioimmunoassay (ng/ml, vertical scale). Each point represents the mean of three experiments. TXB_2 was undetectable in the TPTF-treated platelets.

metabolites is presumed to be exerted on the enzymatic release of arachidonic acid from the platelet phospholipids. Mechanisms for release of arachidonic acid involving phospholipase A_2 [18] or the sequential action of a phosphatidylinositol-specific phospholipase C and diglyceride lipase [19–21] have been proposed.

The release of arachidonic acid from platelet phospholipids is stimulated by a variety of platelet aggregation agents, including collagen. Once arachidonic acid is released from phospholipids, it is then metabolized by fatty acid cyclooxygenase to prostaglandin endoperoxides (prostaglandins G_2 and H_2) that are subsequently converted to prostacyclin, TXA_2, the stable prostaglandins (PGE_2, $PGF_{2\alpha}$ and PGD_2), HHT and MDA. More recently, it has been established that free arachidonic acid can also be converted by lipoxygenase enzyme to HETE [22]. The formations of TXA_2, HHT and MDA from prostaglandin H_2 have been demonstrated to be catalyzed by the same enzyme, TX synthase [23]. Thus, MDA production can be used as a suitable marker for platelet TXA_2 formation.

Perhaps the most interesting observation in the present investigation concerns the specific, inhibitory action of TPTF on platelet aggregation and ATP secretion. Although triphenylarsine and triphenylantimony did not have any effect on platelet aggregation, TPTF almost completely inhibited the platelet aggregation, ATP secretion and MDA formation induced by collagen but not sodium arachidonate.

The inhibitory effect of TPTF on platelet aggregation was apparently different from that of aspirin, which blocked the aggregating activity of both collagen and arachidonic acid. Furthermore, aspirin inhibited the platelet MDA formation induced by both arachidonic acid and collagen. The present results with aspirin are consistent with a previous report [24] indicating that aspirin inhibits TXA_2 formation by blocking cyclooxygenase activity and, thus, preventing second phase platelet aggregation. Chromatographic study showed that the collagen-induced production of arachidonate metabolites (HHT, HETE and TXB_2) from [^{14}C]arachidonic acid-labeled platelets was inhibited markedly by TPTF. Furthermore, TPTF inhibited the collagen-induced production of endogenous TXB_2 from platelets. In contrast, TPTF did not inhibit the platelet aggregation and MDA formation by exogenous arachidonic acid. These results suggested to us that TPTF might inhibit arachidonate release from platelet phospholipids rather than affect the metabolism of arachidonic acid once it was released. We investigated, therefore the release of arachidonic acid from TPTF-treated platelets and confirmed that TPTF remarkably inhibited the release of arachidonic acid at a concentration as low as 10^{-5} M. It thus appears likely the the inhibition of collagen-induced aggregation by TPTF is caused by blocking the release of arachidonic acid from platelet phospholipids.

Arachidonic acid comes from at least two different pathways: phospholipase A_2, acting preferentially on phosphatidylethanolamine and phosphatidylcholine, and phospholipase C, acting on phosphatidylinositol [25]. In the latter pathway, the action of phospholipase C on phosphatidylinositol results in the liberation of 1,2-diacylglycerol [9, 10, 19]. This compound can then be acted upon by diglyceride lipase to form 1-acylglycerol with liberation of arachidonic acid [20]. Metabolism of 1,2-diacylglycerol also results in the liberation of phosphatidic acid [9, 10, 26–28]. Taking into account the two enzymatic pathways triggering arachidonic acid release as men-

tioned above, the results in the present study, that phosphatidic acid formation was not inhibited, indicate that the action of phospholipase C was not inhibited by TPTF. This might account for the trend towards increased arachidonic acid release after 300 sec as depicted in Fig. 4 and the production of arachidonate metabolites as depicted in Fig. 3.

On the other hand, Blackwell *et al.* [29] have shown that mepacrine (quinacrine) has no effect on the velocity of the platelet aggregation induced by arachidonate, although it blocks collagen-induced and thrombin-induced aggregation by direct inhibition of platelet phospholipase A_2. Furthermore, Lapetina *et al.* [28] have shown that mepacrine inhibits the release of arachidonic acid from phospholipids but does not inhibit the conversion of phosphatidylinositol to phosphatidic acid. Considering the similarity of the effects of TPTF to these of mepacrine, it seems likely that TPTF inhibits the liberation of arachidonic acid by acting on phospholipase A_2. Further studies are necessary to investigate whether TPTF, like mepacrine, directly inhibits phospholipase A_2 or not.

Several workers have reported that elevations of platelet cyclic AMP are accompanied by inhibition of aggregation of platelets and of arachidonic acid release from platelet phospholipids [30, 31]. However, the total cyclic AMP content of platelets was not increased by 15 min of incubation with TPTF. This seems to exclude the possibility that the inhibitory effects of TPTF on platelet aggregation and arachidonic acid release are due to the elevation of cyclic AMP content.

With regard to the inhibition by TPTF of ATP secretion from platelets, TPTF also inhibits insulin and glucagon secretion from morphologically intact rabbit islet cells [32]. It seems likely that TPTF affects a common mechanism that initiates the stimulus-induced secretory process in the cells.

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