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Mechanism of Action of *p*-Chlorobiphenyl on the Inhibition of Platelet Aggregation

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Abstract

p-Chlorobiphenyl (1–50 μM) concentration-dependently inhibited the aggregation and release reaction of rabbit washed platelets induced by arachidonic acid and collagen, but not those induced by platelet-activating factor (PAF), U46619 and thrombin. The IC50 values of *p*-chlorobiphenyl on the arachidonic acid and collagen-induced platelet aggregation were 2·9 ± 0·5 and 12·8 ± 2·3 μM, respectively. The formation of both platelet thromboxane B_2 and prostaglandin D_2 caused by arachidonic acid was inhibited by *p*-chlorobiphenyl concentration-dependently. In myo-[^{3}H]inositol-labeled and fura-2-loaded platelets, [^{3}H]inositol monophosphate generation and the rise in intracellular Ca^{2+} stimulated by arachidonic acid were inhibited by *p*-chlorobiphenyl. In human platelet-rich plasma, *p*-chlorobiphenyl and indomethacin prevented the secondary aggregation and blocked ATP release from platelets induced by adenosine 5′-diphosphate and adrenaline without affecting the primary aggregation.

It is concluded that *p*-chlorobiphenyl may be a cyclo-oxygenase inhibitor and its antiplatelet action is mainly due to the inhibition of thromboxane formation.

Platelet aggregation is the main mediator involved in thrombosis and atherosclerosis. At sites of endothelium injury, platelets adhere to the subendothelium, form thromboxane A_2 (TXA_2) and release granule contents (Packham & Mustard 1984). TXA_2 formed from arachidonic acid derived from membrane phospholipids of stimulated platelets is a potent platelet-aggregating agent and vasoconstrictor (Moncada & Vane 1979) and is thought to be responsible for the recruitment of further platelets to the initial aggregates (Svensson et al 1976). In addition, recent studies in-vitro indicate TXA_2 enhances vascular smooth muscle cell proliferation (Uehara et al 1988; Ishimitsu et al 1990; Ko et al 1995). Based on these studies, it seems possible that TXA_2 released from aggregated platelets plays some role in cardiovascular diseases including thrombosis, atherosclerosis, hypertension and arterial restenosis after angioplasty (Ross 1986; Ip et al 1990). Thus, inhibition of platelet function by preventing TXA_2 formation may be a promising approach for the prevention of thrombosis and atherosclerosis.

In a large-scale screening test, we found *p*-chlorobiphenyl was a potent inhibitor of platelet aggregation. In this paper, we have investigated the antiplatelet action of *p*-chlorobiphenyl and attempted to elucidate its mode of action.

Materials and Methods

Platelet preparation

Blood was collected from the rabbit marginal ear vein, or from healthy human volunteers who had taken no medication for 2 weeks before collection. Samples were anticoagulated with sodium citrate (3·8%, 14 : 1) and centrifuged for 10 min at 90 *g* at room temperature (25°C); platelet-rich plasma (PRP) was obtained from the upper portion. The platelet suspension was obtained from EDTA-anticoagulated PRP according to the washing procedure described previously (Teng & Ko 1988). Platelet numbers were counted by Hemalaser 2 (Sebia, France) and adjusted to $4·5 \times 10^8$ platelets mL^{-1}. The platelet pellets were suspended in Tyrode solution containing calcium (1 mM) and bovine serum albumin (0·35%). All glassware was siliconized.

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Platelet aggregation and ATP release

PRP or the platelet suspension was stirred at 900 rev min^{-1} for 1 min and the aggregation inducer was added to trigger the platelet aggregation. Aggregation was measured by the turbidimetric method (O'Brien 1962). The absorbance of PRP or the platelets suspension was taken as 0% aggregation and that of platelet-poor plasma or platelet-free Tyrode solution as 100% aggregation. ATP released from platelets was detected by bioluminescence (DeLuca & McElory 1978). ATP at a known concentration was used to calibrate the intensity of bioluminescence. Both the aggregation and release of ATP were simultaneously measured by a Lumi-aggregometer (Model 1020, Payton, Canada) connected to two dual-channel recorders.

Thromboxane B_2 (TXB_2) and prostaglandin D_2 (PGD_2) assay

Six minutes after the challenge of platelets with the aggregation inducer, 2 mM EDTA and 50 μM indomethacin were added. After centrifugation in an Eppendorf centrifuge (Model 5414) for 2 min, the supernatant was obtained, and TXB_2 and PGD_2 were assayed by enzyme immunoassay (EIA).

Labelling of membrane phospholipids and measurement of the production of [^{3}H]inositol phosphate

This method was modified from those of Huang & Detwiler (1986a) and Neylon & Summers (1987). EDTA-PRP was centrifuged at 500 *g* for 10 min, the platelet pellets were suspended in 700 μL Ca^{2+}-free and BSA-free Tyrode solution containing 75 μCi mL^{-1} [^{3}H]inositol and 1 mM EDTA. After incubation for 2 h at 37°C, the platelets were collected by centrifugation (500 *g*, 4 min) and suspended in fresh Tyrode solution. The reaction was carried out at 37°C for 6 min with 1 mL platelet suspension in a 3·5-mL cuvette with a stirring bar driven at 900 rev min^{-1}. An equal volume of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation at 1000 *g* for 10 min, 1 mL supernatant was pooled and trichloroacetic acid was removed by extracting with 5 × 2 vols diethyl ether. The aqueous phase, containing the inositol phosphate, was adjusted to pH 7–8 and diluted to 4 mL with distilled water before its application to a Dowex-1 ion-exchange column for separation of the inositol phosphates, as described previously by Neylon & Summers (1987). All the experiments were carried out in the presence of 5 mM LiCl to inhibit inositol phosphate phosphatase. Because the levels of inositol bisphosphate and inositol trisphosphate were very low, we measured the inositol monophosphate as an index of the total inositol phosphate formation.

Measurement of intracellular calcium in platelets

The method of Pollock & Rink (1986) was followed. Platelets (4·5 × 10^8 mL^{-1}) were incubated with fura-2/AM (5 μM) at 37°C for 40 min and then centrifuged at 500 *g*; the resultant pellet was washed with EDTA (1 mM)-containing Tyrode solution. After centrifugation, platelets were resuspended in Tyrode solution containing 1 mM Ca^{2+}. Fluorescence (excitation 339 nm, emission 500 nm) was measured with a Hitachi Fluorescence Spectrophotometer (model F4000). At the end of the experiment, the cells were treated with 0·1% Triton X-100 followed by the addition of 10 mM EGTA to obtain the maximal and minimal fluorescence, respectively. $[Ca^{2+}]_i$ was calculated as described for fura-2 using the Ca^{2+}-dye dissociation constant of 224 nM (Grynkiewicz et al 1985).

Materials

p-Chlorobiphenyl was synthesized as follows. *p*-Chlorophenylhydrazine (1·7 g) was dissolved in benzene (100 mL) and lead tetraacetate (8·8 g) was added. The reaction mixture was refluxed for 1 h. After the reaction was completed, the reaction mixture was filtered. The filtrate was washed with water and dried over anhydrous magnesium sulphate and filtered. The benzene solvent was evaporated. The residue was purified by column chromatography (silica gel-petroleum ether) and recrystallized from ether-petroleum ether to yield *p*-chlorobiphenyl (1·61 g, 85% yield). The structure of *p*-chlorobiphenyl was confirmed by spectroscopic methods (UV, IR, [^{1}H]NMR, [^{13}C]NMR, mass spectroscopy (MS) and 2-dimensional NMR).

Collagen (type I, bovine Achilles tendon), obtained from Sigma Chemical Co., St Louis, MO, was homogenized in 25 mM acetic acid and stored at −70°C at a concentration of 1 mg mL^{-1}. Thrombin (bovine) was purchased from Parke Davis & Co., Detroit, MI, and dissolved in 50% glycerol to give a stock solution of 100 NIH units mL^{-1}. Platelet-activating factor (PAF) was purchased from Sigma Chemical Co., dissolved in chloroform and diluted into 0·1% BSA-saline solution immediately before use. ADP, arachidonic acid, EDTA (disodium salt), luciferin-luciferase, dimethylsulphoxide (DMSO), Dowex-1 (100–200 mesh: X8, chloride) resin, myoinositol, indomethacin, U46619 (9,11-dideoxy-9α, 11α-methano-epoxy-PGF$_{2\alpha}$), fura-2/AM and trichloroacetic acid were purchased from Sigma Chemical Co. TXB_2 and PGD_2 EIA kits were purchased from Cayman Chemical Co., Ann Arbor, Michigan. *Myo*[2-^{3}H]inositol was obtained from Amersham (Amersham, UK).

Data analysis

The experimental results are expressed as the means ± s.e.m. Statistical significance was assessed by Student's *t*-test and $P < 0.05$ was considered significant.

Results

Effects of p*-chlorobiphenyl on platelet aggregation and ATP-release reaction*

p-Chlorobiphenyl inhibited the aggregation and ATP release induced by arachidonic acid (100 μM) in a concentration-dependent manner in rabbit washed platelets. The IC50 value of *p*-chlorobiphenyl on arachidonic acid-induced aggregation was 2·9 ± 0·5 μM (Fig. 1, Table 1). At higher concentration ranges, *p*-chlorobiphenyl also inhibited collagen (10 μg mL^{-1})-induced platelet aggregation and ATP release with an IC50 value 12·8 ± 2·3 μM. However, *p*-chlorobiphenyl (50 μM) did not affect the platelet aggregation caused by U46619 (1 μM), PAF (3·6 nM) and thrombin (0·1 units mL^{-1}) (Fig. 1, Table 1). Since *p*-chlorobiphenyl specifically inhibited the effects caused by arachidonic acid, comparison of the concentration–response curves of indomethacin and aspirin were also made. Indomethacin and aspirin inhibited arachidonic acid-induced platelet aggregation in a concentration-dependent manner with IC50 values 0·6 ± 0·1 and 18·9 ± 2·7 μM, respectively (Table 1 for indomethacin). Thus, *p*-chlorobiphenyl was about six and one-fifth as potent as aspirin and indomethacin, respectively. Indomethacin at higher concentrations (10 and 20 μM) also partially suppressed collagen (10 μg mL^{-1})-induced platelet aggregation. Imidazole (1 mM), an inhibitor of thromboxane synthase, did not significantly inhibit arachidonic acid-induced platelet aggregation (Table 1).

In human PRP, adrenaline (5 μM) and ADP (5 μM) caused biphasic aggregation. Secondary aggregation and ATP release were inhibited by *p*-chlorobiphenyl (5–20 μM) in a concentration-dependent manner, while primary aggregation was still not affected at a concentration of 100 μM. Indomethacin (20 μM) also selectively blocked the secondary aggregation and ATP release without affecting the primary aggregation of ADP and adrenaline (Fig. 2).

Effects of p*-chlorobiphenyl on platelet TXB_2 and PGD_2 formation*

The TXB_2 level of resting platelets was 0·5 ± 0·1 ng mL^{-1}. In rabbit platelets, U46619 (1 μM) failed to raise the level of TXB_2 significantly, whereas PAF (3·6 nM) caused only slight

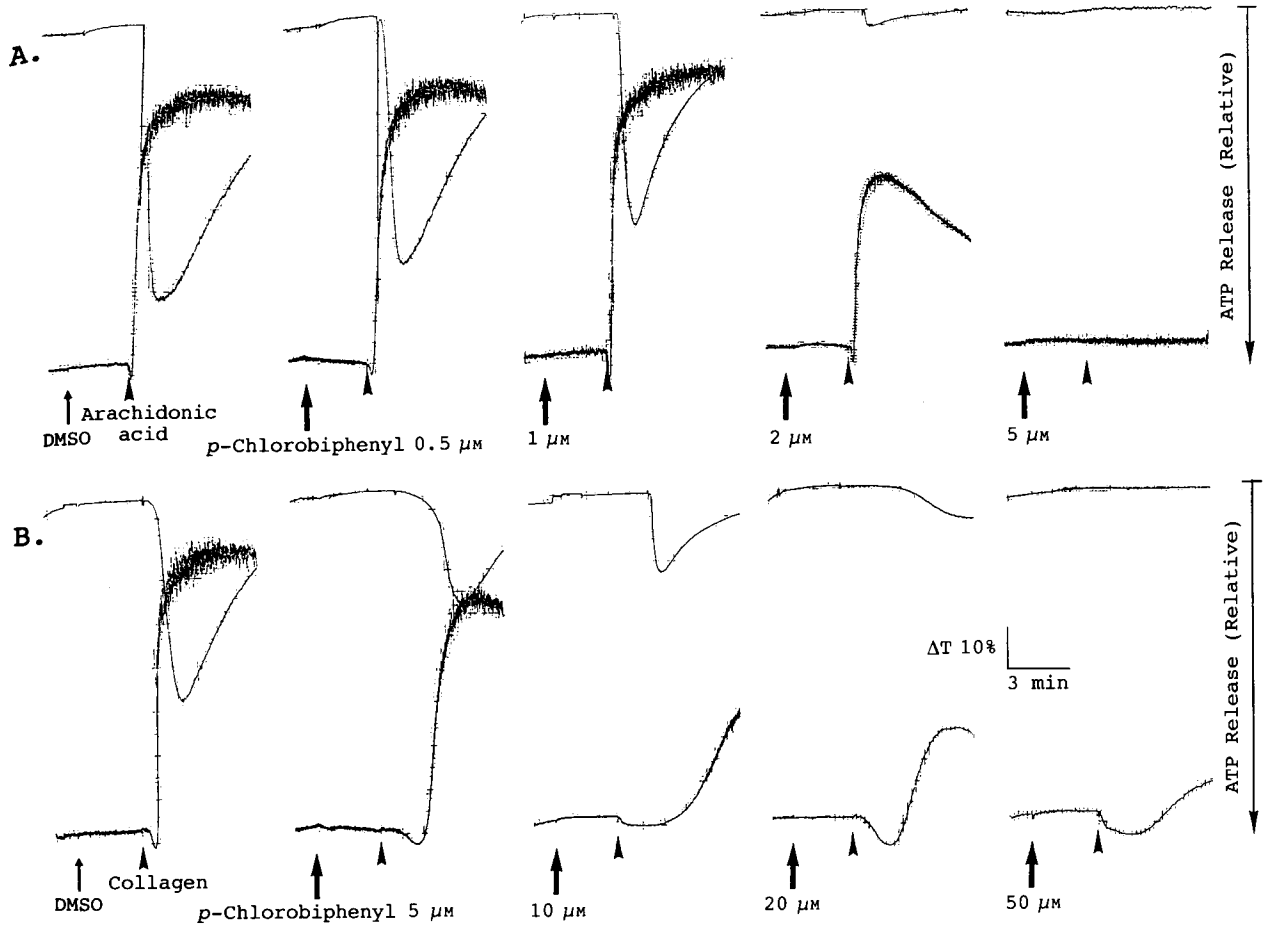

FIG. 1. Inhibitory effects of *p*-chlorobiphenyl on platelet aggregation and ATP release induced by arachidonic acid and collagen. Rabbit washed platelets were incubated with DMSO (0·5%, control) or various concentrations of *p*-chlorobiphenyl at 37°C for 3 min; then arachidonic acid (100 μM, A) or collagen (10 μg mL^{-1}, B) was added to trigger aggregation (upward tracing) and ATP release (downward tracing).

Table 1. Concentration-dependent inhibition of *p*-chlorobiphenyl on arachidonic acid-, collagen-, U46619-, PAF- and thrombin-induced platelet aggregation in rabbit washed platelets.

	Inhibition (%)				
	Arachidonic acid	Collagen	U46619	PAF	Thrombin
p-Chlorobiphenyl					
1 (μM)	1·2 ± 0·7	1·8 ± 1·0	—	—	—
2	33·9 ± 19·1	6·0 ± 3·0	—	—	—
5	100·0 ± 0·0	14·6 ± 7·1	—	—	—
10	—	48·1 ± 12·5	—	—	—
20	—	64·0 ± 8·5	—	—	—
50	—	82·3 ± 7·4	7·2 ± 3·0	0·6 ± 0·5	1·1 ± 1·0
Indomethacin					
0·2 (μM)	3·1 ± 1·2	—	—	—	—
0·5	41·7 ± 12·6	—	—	—	—
1·0	100·0 ± 0·0	—	—	—	—
10	—	59·8 ± 10·4	—	—	—
20	—	59·6 ± 13·8	—	—	—
Imidazole					
1 (mM)	6·3 ± 1·5	—	—	—	—

Rabbit washed platelets were preincubated with DMSO (0·5%, control) or various concentrations of *p*-chlorobiphenyl at 37°C for 3 min, then arachidonic acid (100 μM), collagen (10 μg mL^{-1}), U46619 (1 μM), PAF (3·6 nM) or thrombin (0·1 NIH units mL^{-1}) was added to trigger aggregation. Values are means ± s.e.m. (n = 4–6).

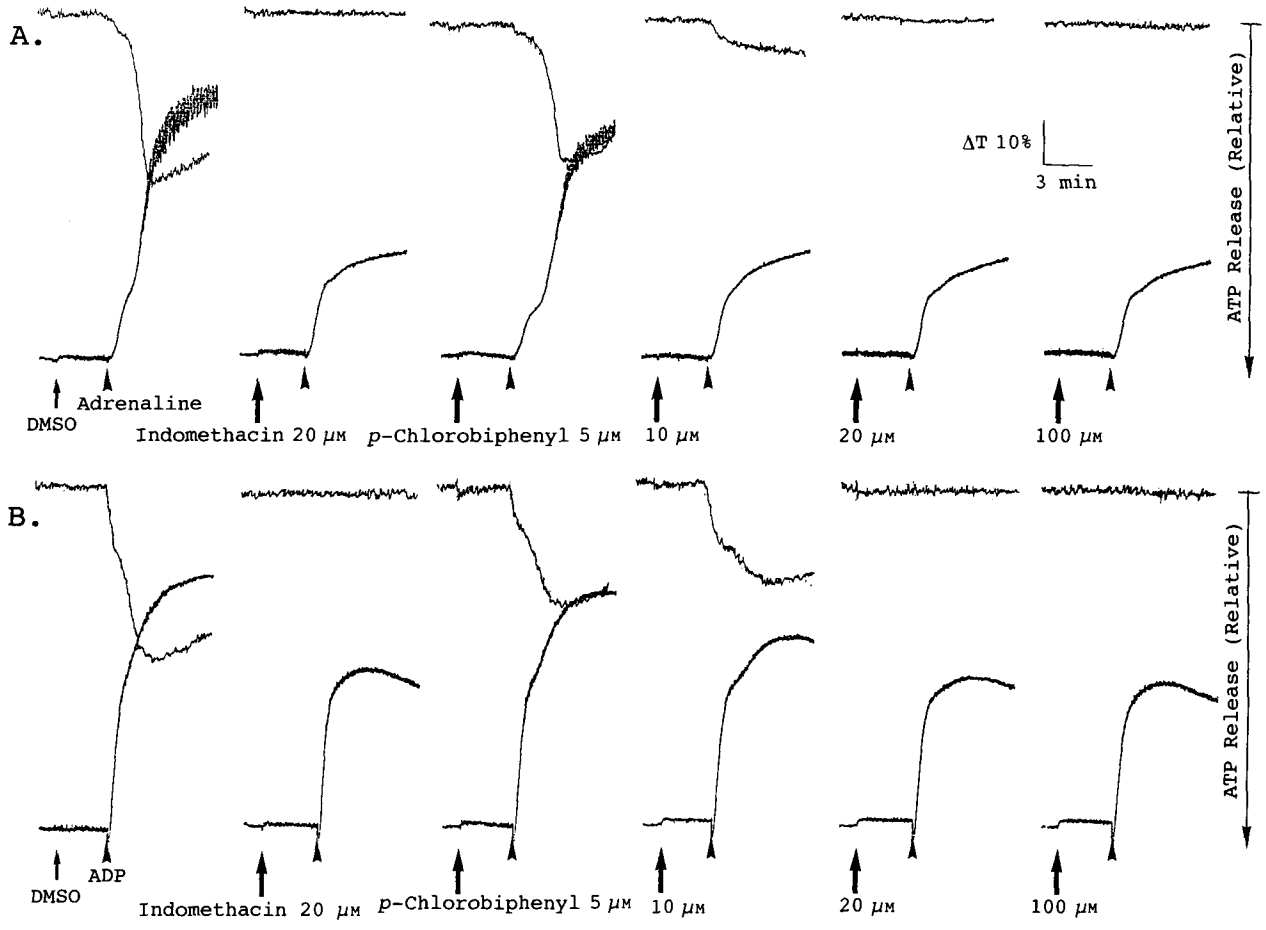

FIG. 2. Effects of *p*-chlorobiphenyl on the aggregation and ATP release induced by adrenaline and ADP in human PRP. PRP was incubated with DMSO (0·5%, control), indomethacin (20 μM) or various concentrations of *p*-chlorobiphenyl at 37°C for 3 min; then adrenaline (5 μM, A) or ADP (5 μM, B) was added to trigger aggregation (upward tracing) and ATP release (downward tracing).

thromboxane formation. However, arachidonic acid (100 μM), collagen (10 μg mL^{-1}) and thrombin (0·1 NIH units mL^{-1}) caused marked TXB_2 formation in rabbit washed platelets. *p*-Chlorobiphenyl significantly inhibited arachidonic acid-, collagen-, PAF- and thrombin-induced TXB_2 formation. Indomethacin (0·5 μM) and imidazole (1 mM) also inhibited arachidonic acid-induced TXB_2 formation significantly (Table 2). The PGD_2 level of

Table 2. Effects of *p*-chlorobiphenyl, indomethacin and imidazole on the thromboxane B_2 formation in rabbit washed platelets caused by arachidonic acid, collagen, U46619, PAF and thrombin.

	Thromboxane B_2 (ng mL^{-1})				
	Arachidonic acid	Collagen	U46619	PAF	Thrombin
Control	684 ± 116	278 ± 73	0·6 ± 0·1	10 ± 4	28 ± 4
p-Chlorobiphenyl					
1 (μM)	52 ± 6**	—	—	—	—
2	39 ± 15***	31 ± 14*	—	—	—
5	17 ± 6***	20 ± 9**	0·5 ± 0·1	0·5 ± 0·1*	2·5 ± 1***
50	—	5 ± 4**	0·6 ± 0·1	0·6 ± 0·1*	0·4 ± 0·1***
Indomethacin					
0·5 (μM)	9 ± 3***	—	—	—	—
Imidazole					
1 (mM)	44 ± 9***	—	—	—	—

DMSO (0·5% control), indomethacin, imidazole or various concentrations of *p*-chlorobiphenyl was preincubated with platelets at 37°C for 3 min; then arachidonic acid (100 μM), collagen (10 μg mL^{-1}), U46619 (1 μM), PAF (3·6 nM) or thrombin (0·1 NIH units mL^{-1}) was added. Aggregation and thromboxane formation were terminated by EDTA (2 mM) and indomethacin (50 μM) 6 min after the addition of the inducer. Values are means ± s.e.m. (n = 4–6). *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$ as compared with the respective control.

Table 3. Effects of *p*-chlorobiphenyl, indomethacin and imidazole on prostaglandin D_2 formation induced by arachidonic acid in rabbit washed platelets.

	Prostaglandin D_2 ($pg\,mL^{-1}$)
Resting	4 ± 1
DMSO + arachidonic acid	355 ± 60
p-Chlorobiphenyl (2 μM) + arachidonic acid	29 ± 9***
(5 μM) + arachidonic acid	9 ± 2***
Indomethacin (0·5 μM) + arachidonic acid	13 ± 2***
Imidazole (1 mM) + arachidonic acid	2107 ± 462**

DMSO (0·5%), *p*-chlorobiphenyl, indomethacin or imidazole was preincubated with platelets at 37°C for 3 min; then arachidonic acid (100 μM) was added. Aggregation and prostaglandin D_2 formation were terminated by EDTA (2 mM) and indomethacin (50 μM) 6 min after the addition of arachidonic acid. Values are means ± s.e.m. (n = 4–5). $^{**}P < 0.01$; $^{***}P < 0.001$ as compared with the control (arachidonic acid, 100 μM).

resting platelets was low (4 ± 1 $pg\,mL^{-1}$). However, PGD_2 formed in the presence of arachidonic acid. This PGD_2 formation was inhibited by both *p*-chlorobiphenyl and indomethacin, but enhanced markedly by imidazole (1 mM) (Table 3).

Effects of p-*chlorobiphenyl on phosphoinositide breakdown*

In [^{3}H]inositol-labelled rabbit washed platelets, collagen (10 $\mu g\,mL^{-1}$), U46619 (1 μM), PAF (3·6 nM) and thrombin (0·1 NIH units mL^{-1}) increased [^{3}H]inositol monophosphate formation 2·7 ± 0·3-, 2·9 ± 0·3-, 3·2 ± 0·4- and 5·4 ± 0·4-fold, respectively, in the presence of indomethacin (2 μM). Arachidonic acid (100 μM), in the absence of indomethacin, also increased [^{3}H]inositol monophosphate formation 2·1 ± 0·2-fold. *p*-Chlorobiphenyl (5 μM) almost completely inhibited arachidonic acid-induced [^{3}H]inositol monophosphate formation. However, a high concentration of *p*-chlorobiphenyl (50 μM) did not affect this formation caused by collagen, U46619, PAF and thrombin.

Effects of p-*chlorobiphenyl on the intracellular calcium of platelets*

In fura-2-loaded platelets, collagen (10 $\mu g\,mL^{-1}$), U46619 (1 μM), PAF (3·6 nM) and thrombin (0·1 NIH units mL^{-1}) caused an increase of intracellular free calcium in the presence of indomethacin (2 μM). Arachidonic acid (100 μM) also increased intracellular free calcium in the absence of indomethacin. The rise was only short-lived, and apparent intracellular free calcium declined towards the resting level within a few minutes. This was due to the aggregates interfering with the fluorescence signal and constitutes a limitation of the technique. As shown in Fig. 3, only the increase of intracellular free calcium caused by arachidonic acid was inhibited by *p*-chlorobiphenyl.

Discussion

The present study shows that *p*-chlorobiphenyl is an inhibitor of platelet aggregation and ATP release induced by arachidonic acid and collagen. Its mechanism of action may be due to inhibition of platelet cyclo-oxygenase activity.

TXA_2 is an important mediator of release reaction and aggregation of platelets (Hornby 1982). The aggregation and ATP release of platelets induced by arachidonic acid are due to TXA_2 formation (Hamberg et al 1975). Exogenous addition of arachidonic acid is converted by platelet cyclo-oxygenase to the prostaglandin endoperoxides, which in

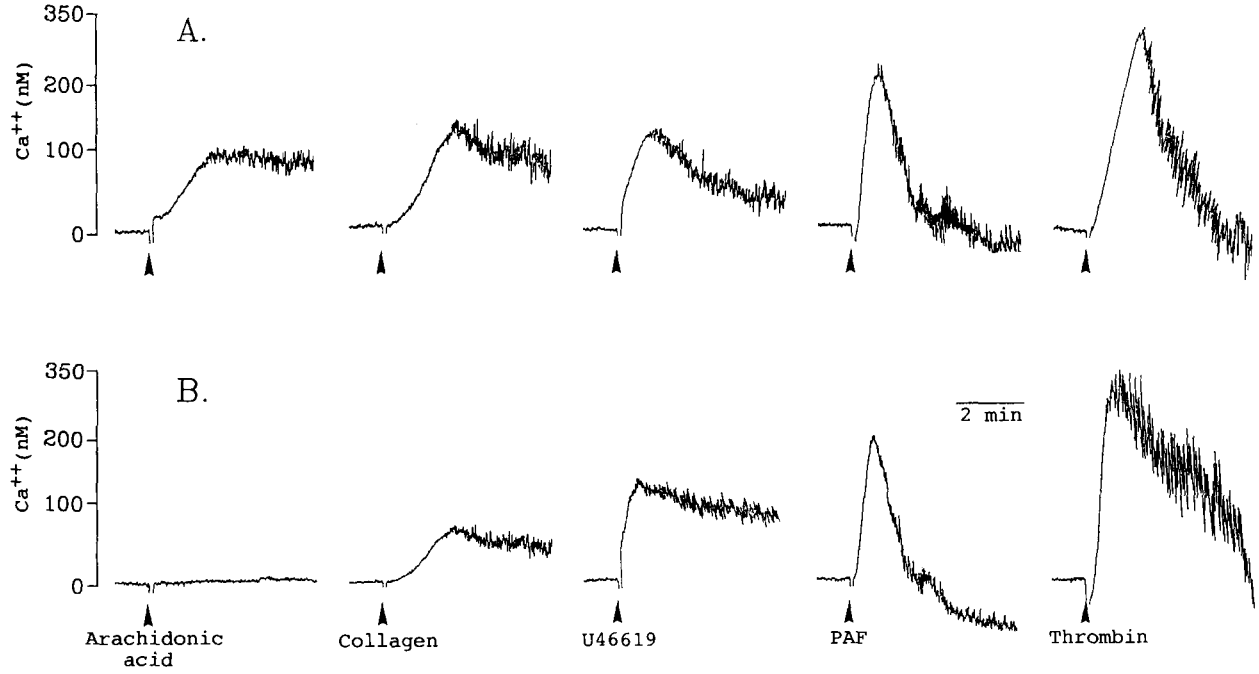

FIG. 3. Effects of *p*-chlorobiphenyl on the increase of intracellular calcium concentration caused by some aggregation inducers in fura-2-loaded platelets. Fura-2-loaded rabbit washed platelets were preincubated with DMSO (0·5%, A) or *p*-chlorobiphenyl (5 μM for arachidonic acid; 50 μM for other inducers; B) at 37°C for 3 min; then arachidonic acid (100 μM), collagen (10 $\mu g\,mL^{-1}$), U46619 (1 μM), PAF (3·6 nM) or thrombin (0·1 NIH. units mL^{-1}) was added. Indomethacin (2 μM) was present in the medium except in that experiment challenged by arachidonic acid.

turn are converted by thromboxane synthase to TXA_2. In human PRP, ADP and adrenaline cause biphasic aggregation with secretion accompanying the second phase. Secondary aggregation and ATP release are mediated by TXA_2 formation and inhibited by cyclo-oxygenase inhibitors, such as aspirin (Huang & Detwiler 1986b). *p*-Chlorobiphenyl inhibited arachidonic acid-, collagen-, PAF- and thrombin-induced TXA_2 formation. It also inhibited arachidonic acid-induced phosphoinositide breakdown and the rise of intracellular calcium concentration. In human PRP, *p*-chlorobiphenyl inhibited only secondary aggregation and ATP release without affecting the primary aggregation of ADP and adrenaline. These results indicate that *p*-chlorobiphenyl inhibits platelet aggregation and release reaction via inhibition of TXA_2 formation. Similar to indomethacin, but not to imidazole, *p*-chlorobiphenyl inhibited PGD_2 formation in the presence of arachidonic acid. Thus, *p*-chlorobiphenyl may be an inhibitor of cyclo-oxygenase. The action of *p*-chlorobiphenyl is different from that of a TXA_2 receptor antagonist, such as SQ29548, which fails to alter cyclo-oxygenase, thromboxane synthase or adenylate cyclase activity (Ogletree et al 1985). Furthermore, *p*-chlorobiphenyl did not inhibit U46619-induced platelet responses indicating that it is not a TXA_2-receptor antagonist.

Phosphoinositide breakdown is observed in platelets activated by many agonists, and may be a primary event in agonist-induced activation (Berridge 1984; Nishizuka 1984). This phosphoinositide breakdown in platelets may provide a source of free arachidonic acid via the diglyceride lipase pathway or phosphatidic acid-specific phospholipase A_2 leading to TXA_2 formation (Bell et al 1979; Billah et al 1981). Arachidonic acid also can be liberated from membrane phospholipids by phospholipase A_2, which is activated by inositol 1,4,5-trisphosphates-triggered calcium release (McKean et al 1981). In the presence of indomethacin, to abolish the further formation of TXA_2, the phosphoinositide breakdown and rise of intracellular free calcium caused by collagen, U46619, PAF and thrombin were not inhibited by the addition of *p*-chlorobiphenyl (50 μM). In contrast, the phosphoinositide breakdown and rise of intracellular free calcium induced by arachidonic acid were markedly inhibited by *p*-chlorobiphenyl (5 μM). The inhibition of arachidonic acid metabolism and TXA_2 formation rather than the direct inhibition on phosphoinositide breakdown, was thus responsible for the failure of blockade on phosphoinositide breakdown caused by collagen, U46619, PAF and thrombin.

In conclusion, *p*-chlorobiphenyl may be a cyclo-oxygenase inhibitor and its antiplatelet action is mainly due to the inhibition of thromboxane formation.

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