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EFFECTS OF TWO METHYLXANTHINES, PENTOXIFYLLINE AND PROPENTOFYLLINE, ON ARACHIDONIC ACID METABOLISM IN PLATELETS STIMULATED BY THROMBIN

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Abstract—[^{3}H]Pentoxifylline and [^{3}H]propentofylline were taken up by human platelets in a dose-dependent manner probably involving a passive diffusion through the plasma membrane. *In vitro*, the two drugs were able to inhibit platelet activation induced by thrombin. Serotonin secretion was reduced from 57% to 38% and 28% in the presence of 1 mM pentoxifylline and 1 mM propentofylline, respectively. Platelet aggregation was inhibited in the same way. Modifications of [^{14}C]arachidonic acid metabolism in human platelets stimulated by thrombin were then measured in the presence of drugs. Preincubation of platelets with 1 mM pentoxifylline or propentofylline inhibited the production of [^{14}C]arachidonic acid metabolites, without any accumulation of free arachidonic acid, suggesting an action at a step preceding its conversion. Phosphatidylinositol and phosphatidylcholine hydrolysis measured upon thrombin treatment as well as phosphatidic acid production were reduced or suppressed in the presence of the drugs. A dose-dependence study showed that phosphatidylcholine hydrolysis was totally inhibited at 5.10^{-4} M propentofylline, while phosphatidic acid formation was reduced by only 40%. Propentofylline was in general more efficient than pentoxifylline in inhibiting events occurring upon thrombin stimulation. Our results suggest that the two methylxanthines inhibit both phospholipase A_2 and phospholipase C, the former displaying a greater sensitivity to the two drugs.

Pentoxifylline (3,7-dimethyl-1-(5-oxo-hexyl)-xanthine), (Trental, Hoechst AG, Frankfurt/M., F.R.G.) is widely used for the treatment of peripheral vascular disease. Its pharmacological properties seem to be due at first to an increase in erythrocyte flexibility [1], but an *in vivo* inhibition of platelet aggregability, which could participate to the therapeutic action of this drug, was also described [2, 3]. The metabolism of arachidonic acid (AA)* has been largely studied in platelets. Activation of phospholipase C is one of the primary events evoked by aggregating agents such as thrombin [4, 5], platelet activating factor [6–8], thromboxane A_2 [9, 10] or collagen [11]. The first substrate of this enzyme is phosphatidylinositol 4,5-bisphosphate (PIP_2), while hydrolysis of phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol (PI) occurs as a secondary event [4, 5, 12]. This leads to the formation of diglycerides (DG) containing almost exclusively AA in the 2 position of *sn*-glycerol, which can then be released by the action of a plasma membrane lipase [13]. The second product of PIP_2 hydrolysis by phospholipase C, inositol-1,4,5 trisphosphate, is able to mobilize Ca^{2+} from internal stores [14–18]. The increase of cytosolic free Ca^{2+} then activates various enzymes including phospholipase A_2, which liberates AA from phosphatidylcholine (PC) and (or) phosphatidylethanolamine (PE) [19–21]. AA is then metabolized by cyclooxygenase and lipoxygenase into thromboxane A_2 (a powerful aggregating product) and 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), respectively. Looking for a mechanism of action of pentoxifylline on platelet aggregation, we first investigated the ability of this drug to penetrate into cells. In parallel, we compared its behaviour to that of a new structural analog, propentofylline (1-(5-oxohexyl)-3-methylpropylxanthine) which is somewhat more lipophilic owing to its lateral propyl chain. We then investigated the effects of the two drugs on AA metabolism in human platelets prelabelled with [U-^{14}C]AA and stimulated with thrombin. Finally, because of the well known property of methylxanthines to inhibit cyclic AMP phosphodiesterase, cAMP level in platelet stimulated or not with thrombin was determined and synergistic effects of the two drugs with prostaglandin E_1, a direct stimulant of adenylcyclase, were investigated.

* Abbreviations used: AA, arachidonic acid; DG, diglyceride; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PS, phosphatidylserine; PRP, platelet-rich plasma; TXB_2, thromboxane B_2; 12-HETE, 12-L-hydroxy-5,8,10,14-icosatetraenoic acid; HHT, 12-L-hydroxy-5,8,10,heptadecatrienoic acid.

MATERIALS AND METHODS

Human thrombin and prostaglandin E_1 were purchased from Sigma (St Louis, MO). [U-^{14}C] sucrose

(350 mCi/mmol), 5-hydroxy (G-^{3}H) tryptamine creatinine sulfate ([^{3}H] serotonin, 1 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, U.K.). [U-^{14}C] arachidonic acid (10 Ci/mmol) was from New England Nuclear. 3.7 Dimethyl-1-(oxohexyl) xanthine (pentoxifylline) and 3-methyl-7-propyl-1-(oxohexyl) xanthine (propentofylline) were a kind gift from Hoechst, France. Both drugs were also purchased after [^{3}H] labelling and displayed specific radioactivity of 42 Ci/mmole and 36 Ci/mmole, respectively.

Interaction of [^{3}H] *pentoxifylline and* [^{3}H] *propentofylline with platelets.* Platelets from one concentrate were isolated and washed by the method of Ardlie *et al.* [22] and finally suspended in the late washing buffer, without Ca^{2+} (pH 7.35) at a concentration of 10^{10} cells/ml. Platelets were incubated at 37° for 15 min with (U-^{14}C) saccharose (0.1 μCi/ml), [^{3}H] pentoxifylline, [^{3}H] propentofylline (0.5 μCi) and increasing concentrations of non-labelled drug. Platelets were then pelleted in an Eppendorf centrifuge at 15000 rpm for 2 min. [^{3}H] and [^{14}C] radioactivities were both measured in supernatant and pellet, after addition of perchloric acid (10%) to the pellet. The [^{3}H]/[^{14}C] ratio in the supernatants allowed us to calculate [^{3}H] label corresponding to the intercellular part of the total radioactivity found in the pellets. It was then possible to estimate the amount of [^{3}H] radioactivity bound to the cells. This method was based on the fact that [^{14}C]saccharose does not penetrate into the cells.

Preparation of platelet suspensions from fresh human blood. For experiments dealing with functional studies, AA metabolism or cAMP determination, platelets were prepared from fresh human blood, according to the previous procedures [22]. In that case, blood was drawn by venipuncture onto acid citrate dextrose of Aster and Jandl [23] from normal human volunteer donors free of any medication for at least one week. Platelet-rich plasma (PRP) was obtained by centrifugation at 120 *g* for 15 min (20°).

Platelet aggregation and serotonin release. PRP was incubated with [^{3}H]serotonin (200 nCi/ml PRP) for 30 min at 37°. Platelets were then centrifuged at 1000 *g* for 15 min, washed twice and resuspended in Tyrode buffer (pH 7.35) containing 1 mM $MgCl_2$), no calcium and no albumin, at 0.5 10^9 cells/ml. Cells were incubated with 1 U/ml thrombin, following a preincubation with drugs for 15 min at 37°. Incubations were stopped after 2 min by addition of 20 mM EDTA, followed immediately by cooling on ice. Platelets were then pelleted to allow quantitation of [^{3}H]serotonin released from platelet dense granules. Radioactivity was determined by liquid scintillation counting. Platelet aggregation was measured at 37° by the turbidimetric method of Born [24].

Preparation of radiolabelled platelet suspension for phospholipid metabolism-cAMP assay. PRP was sedimented at 1000 *g* for 15 min and suspended in 0.5 vol. (compared to PRP) of a calcium-free Tyrode buffer (pH 6.4) containing 2 mM $MgCl_2$, 5.5 mM glucose, 0.35% bovine serum albumin (w/v) and 0.2 mM EGTA. Platelets were incubated at 37° for 90 min in the presence of [U-^{14}C] AA (0.1 μCi/ml). After a wash in the same buffer lacking EGTA, they were finally suspended in a Tyrode buffer (pH 7.35) containing 1 mM $MgCl_2$, 5.5 mM glucose and 1 mM $CaCl_2$ without bovine serum albumin, at a concentration of 0.5 10^9 cells/ml for phospholipid metabolism and 2.10^8 cells/ml for cAMP assay. The 15 min preincubation in the absence or in the presence of various concentrations of pentoxifylline or propentofylline was followed by addition of thrombin (1 unit/ml, final concentration) for 5 min. In the case of lipid analysis, incubations were stopped by EDTA 20 mM and chloroform/methanol (1/1, v/v). For cAMP assay, reactions were blocked by addition of 3.5 M perchloric acid. The protein precipitate was eliminated by centrifugation. The supernatant was neutralized to pH 6–7 with 9 M KOH and acetylated [25]. Cyclic AMP was assayed using a commercial radioimmunoassay kit (Institut Pasteur, Paris, France).

Lipid analysis. Lipids were extracted according to Bligh and Dyer [26]. After recovering the first chloroformic phase, the medium was acidified with concentrated formic acid (20 μl/ml platelet suspension) and submitted to a second extraction with chloroform [27]. The two lipid extracts were pooled, taken to dryness under nitrogen, dissolved in a minimal volume of chloroform/methanol (1/1, v/v) and submitted to two different chromatographic separations on thin layer plates using the following solvents: (1) chloroform/methanol/acetic acid/water (65/43/1/3, v/v) for the separation of phospholipids [28]; (2) the upper phase of ethylacetate/acetic acid/isooctane/water (110/20/50/100, v/v) which allowed separation of phosphatidic acid, cyclooxygenase and lipoxygenase derivatives of arachidonic acid, non-converted arachidonic acid, diacylglycerol, from the bulk of phospholipids [29]. The various lipid spots were detected by autoradiography and then scraped directly into scintillation vials containing 10 ml of liquid scintillation counting.

Analytical methods. Lactate dehydrogenase was measured as previously described [30] using the method of Wroblewski and La Due [31]. Radioactivity was determined with a Packard-Tricarb 4530 spectrometer equipped with automatic quenching correction using Instagel (Packard, U.S.A.) as scintillation fluid.

RESULTS

Interaction of pentoxifylline and propentofylline with platelets

In some preliminary experiments, platelet suspensions were incubated in the presence of ^{3}H-labelled drugs, followed by several washings. Under these conditions, platelets did not bind significant amounts of the radioactive compounds, which were eliminated by washing, suggesting a loose interaction of the drugs with the cells. However, we could not exclude a passive diffusion through the membrane allowing a rapid equilibration between intracellular and extracellular compartments. To test this point, the radioactivity in the cell pellets was determined following incubation with [^{3}H] drugs before any washing, [^{14}C]sucrose being used to determine the extracellular space of the pellet. By this procedure,

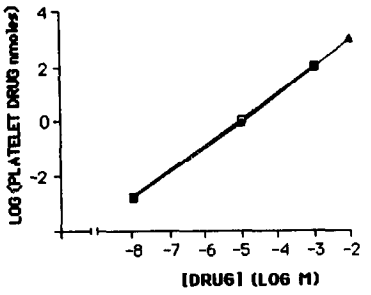

Fig. 1. Interaction of [^{3}H] pentoxifylline (—▲—) and [^{3}H] propentofylline (—□—) with platelets. Washed platelets (10^{10} cells/ml) were incubated with increasing concentrations of the two [^{3}H] labelled drugs and [^{14}C] saccharose for 15 min at 37°. Assays were then processed as described under Materials and Methods. Results are expressed in platelet drug nmol as a function of drug concentration (log/log).

binding and (or) penetration of the two drugs were found to be proportional to their concentration in the external medium (Fig. 1). Respectively 10% and 13% of the total ^{3}H-radioactivity of pentoxifylline and propentofylline was bound to platelets, whatever the concentration of the drug in the medium. Time course studies between 15 sec and 15 min showed that the amount of radioactivity bound to platelets was constant starting from 15 sec (results not shown).

Effects of pentoxifylline and propentofylline on release reaction and aggregation of platelets

Table 1 shows that [^{3}H]serotonin release from thrombin stimulated platelets was inhibited by propentofylline at concentrations equal to or above

Table 1. Effects of pentoxifylline and propentofylline on the release of [^{3}H]serotonin in thrombin-activated platelets

Drug concentration (M)	0	10^{-4}	10^{-3}	10^{-2}
Thrombin + pentoxifylline	57 ± 5	58 ± 6	38 ± 11	9 ± 3
Thrombin + propentofylline	57 ± 5	50 ± 6	28 ± 7	N.D.

Washed platelets prelabelled with [^{3}H]serotonin were incubated in the absence or in the presence of drugs for 15 min and stimulated with thrombin (1 unit/ml) for 5 min at 37°, as described under Materials and Methods. Release is exposed as percentage of total platelet [^{3}H]serotonin and results are presented as means ± SEM of 3 experiments. (N.D., not determined).

10^{-4} M. Higher concentrations of pentoxifylline were necessary to obtain an identical inhibition rate. Under the same experimental conditions, platelet aggregation was inhibited by the two drugs (Fig. 2). Total inhibition was obtained at 10^{-3}M propentofylline and 10^{-2} M pentoxifylline. Measurement of lactate dehydrogenase in platelet supernatants incubated under identical conditions with the two drugs indicated that no lysis occurred at concentrations up to 10^{-3} M propentofylline and 10^{-2} M pentoxifylline. So, inhibition of platelet function promoted by the drugs was not related to cell damage, even at the highest concentrations used in this study.

Effects of drugs on AA metabolism

After exposure to thrombin, [U-^{14}C] labelled platelets produced significant amounts of radioactive cyclooxygenase metabolites, thromboxane B_2 (TXB_2) and 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT), as well as 12-HETE, a 12-lipoxygenase

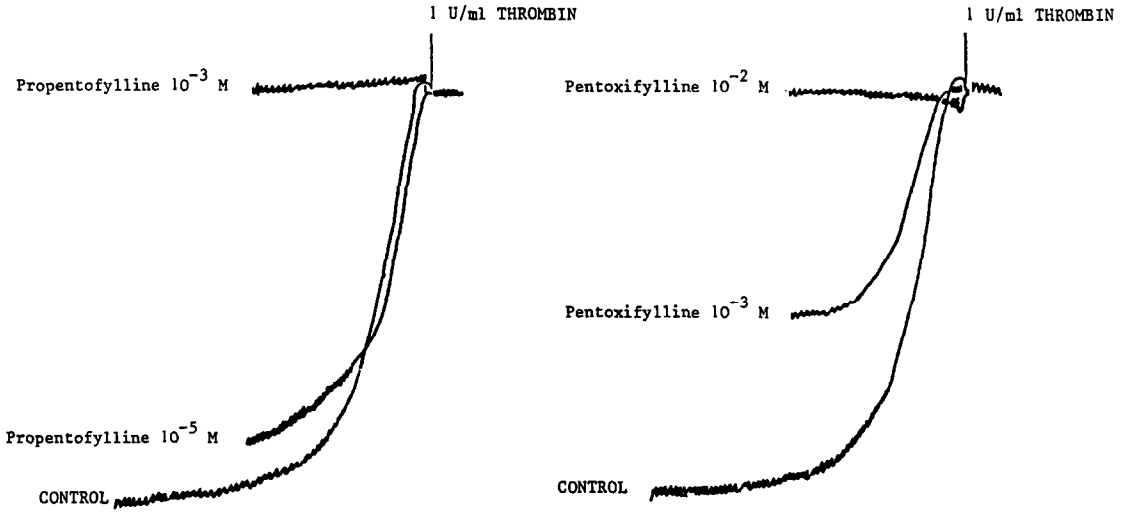

Fig. 2. Inhibition by pentoxifylline and propentofylline of platelet aggregation induced by thrombin (1 unit/ml). Aggregation studies were performed using washed human platelets (0.5 10^9 cells/ml) at 37°, under magnetic stirring. When present, drugs were preincubated for 15 min at 37° before thrombin addition.

Table 2. Effects of pentoxifylline and propentofylline on the distribution of [U-^{14}C]arachidonate radioactivity in arachidonic acid and its metabolites present in platelets, upon thrombin treatment (1 unit/ml)

	Control (N = 5)	Thrombin (N = 5)	Thrombin + Pentoxifylline 1 mM (N = 3)	Thrombin + Pentoxifylline 10 mM (N = 3)	Thrombin + Propentofylline 1 mM (N = 5)
Thromboxane B_2	0.14 ± 0.06	2.42 ± 0.57**	1.66 ± 0.74	0.41 ± 0.18*	0.44 ± 0.08**
H.H.T.	0.10 ± 0.03	2.41 ± 0.57**	1.56 ± 0.68	0.12 ± 0.05*	0.44 ± 0.1**
12-HETE	0.26 ± 0.10	3.17 ± 0.36***	2.43 ± 0.75	0.28 ± 0.18**	1.66 ± 0.64*
Arachidonic acid	0.42 ± 0.08	0.50 ± 0.03	0.54 ± 0.14	0.31 ± 0.12	0.53 ± 0.09

Assays were performed as described under Materials and Methods.
Data are percentages of total lipid radioactivity. (Means ± SEM of N = 3–5 experiments).
Statistical analysis was made according to impaired Student's test: * $P < 0.05$; ** < 0.01; *** < 0.001.
Thrombin assay was compared to control whereas drug effects were measured comparatively to thrombin assay.

Table 3. Effects of pentoxifylline on thrombin-induced modifications in ([U-^{14}C] arachidonate labelling of phospholipids

	Control	Thrombin	Thrombin + Pentoxifylline 1 mM	Thrombin + Pentoxifylline 10 mM
Phosphatidylcholine	48.3 ± 2.3	44.9 ± 2.2**	46.8 ± 2.5	48.4 ± 2.3*
Phosphatidylethanolamine	11.0 ± 1.5	11.4 ± 1.1	10.5 ± 1.0	9.6 ± 0.9
Phosphatidylserine	8.7 ± 0.6	8.5 ± 0.7	8.4 ± 0.8	8.6 ± 0.8
Phosphatidylinositol	24.3 ± 5.7	11.9 ± 1.5*	14.0 ± 1.7*	21.4 ± 3.9*
Phosphatidic acid	0.6 ± 0.1	3.9 ± 0.9*	2.4 ± 0.5	0.7 ± 0.1*
Neutral lipids	7.1 ± 2.8	18.6 ± 2.4*	17.1 ± 3.4	11.1 ± 3.4*

Results are percentages of total radioactivity (mean ± SEM from 4 experiments).
Statistical analysis was made according to paired Student's test: * $P < 0.05$; ** $P < 0.02$.
Thrombin assay was compared to control whereas drug effects were measured comparatively to thrombin assay.

product (Table 2). These changes were significantly depressed by 10^{-2} M pentoxyfylline or 10^{-3} M propentofylline, inhibition attaining 80–100%. In all of these cases, the decrease of [^{14}C]icosanoids was not accompanied by an accumulation of [^{14}C]arachidonic acid, suggesting that the drugs were acting at the step of AA liberation. Indeed, as shown in Tables 3 and 4, analysis of platelet [^{14}C] phospholipids indicated that thrombin induced a significant decrease in the radioactivity of PI and PC, together with a significant increase of phosphatidic acid (PA) and neutral lipids. Here again, these changes were significantly inhibited by 10^{-2} M pentoxifylline (Table 3) or 10^{-3} M propentofylline (Table 4). This inhibitory effect was only detectable in stimulated platelets, since drugs added to resting cells did not modify the distribution of [^{14}C]AA among lipids (not shown).

Figure 3 shows the dose dependent inhibition by pentoxifylline and propentofylline of the various

Table 4. Effects of propentofylline on thrombin-induced modifications in [U^{14}C]arachidonate labelling of phospholipids

	Control	Thrombin	Thrombin + propentofylline 1 mM
Phosphatidylcholine	43.8 ± 3.2	40.2 ± 3.2**	44.8 ± 2.7*
Phosphatidylethanolamine	10.1 ± 1.1	11.2 ± 0.6	9.8 ± 0.8
Phosphatidylserine	8.1 ± 0.6	8.3 ± 0.6	8.3 ± 0.7
Phosphatidylinositol	20.0 ± 4.5	12.2 ± 2.5*	15.3 ± 1.8**
Phosphatidic acid	0.9 ± 0.4	4.1 ± 0.6**	1.7 ± 0.3**
Neutral lipids	17.1 ± 6.5	23.5 ± 5.6*	19.9 ± 4.6*

Results are expressed as percentages of total radioactivity (mean ± SEM of 6 experiments).
Statistical analysis was made according to paired Student's test: * $P < 0.05$; ** $P < 0.01$.
Thrombin assay was compared to control whereas drug effects were measured comparatively to thrombin assay.

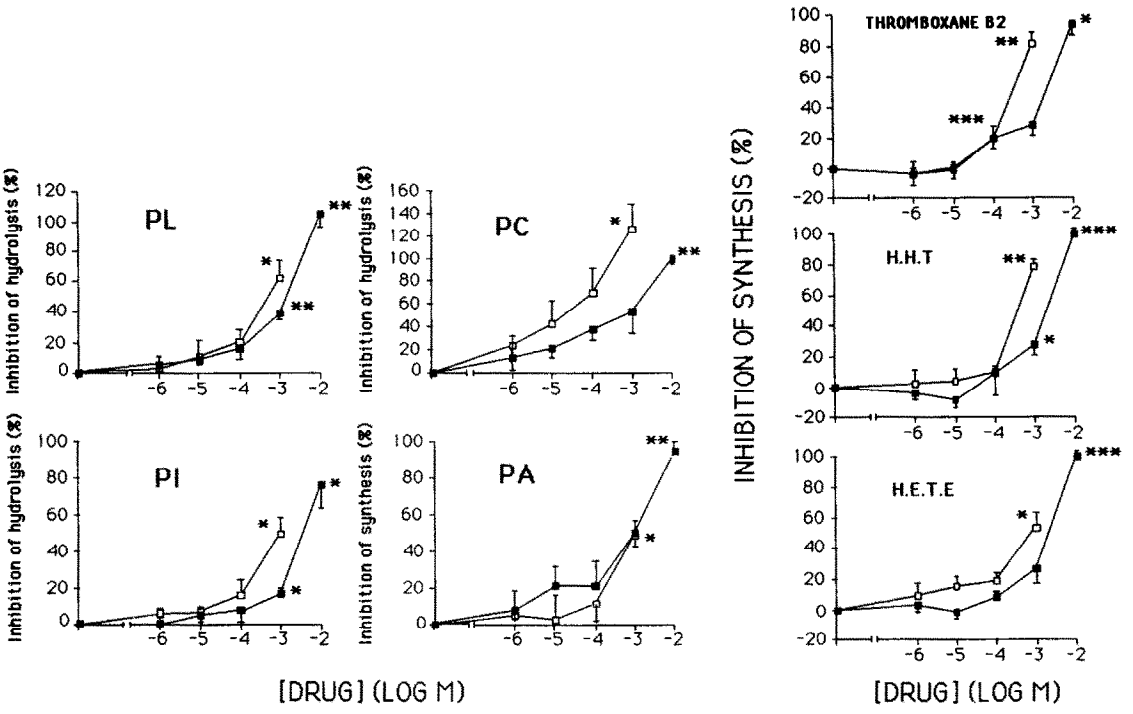

Fig. 3. Effects of increasing pentoxifylline (—■—) and propentofylline (—□—) concentrations on [^{14}C]arachidonate radioactivity changes in the various lipids of human platelets upon thrombin stimulation. Experimental conditions were the same as in Tables 2 and 3. Data correspond to inhibition percentages of variations measured in the radioactivity of the different lipids in the presence of drugs versus modifications induced by thrombin alone. Results are means ± SEM from 4 and 3 experiments, respectively, for pentoxifylline and propentofylline, P, probability of significance according to paired Student's test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (a) Corresponds to total phospholipids (PL), phosphatidylinositol (PI), phosphatidic acid (PA) and phosphatidylcholine (PC) and (b) to arachidonic acid metabolites (12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE); thromboxane B_2 and 12-L-hydroxy-5,8,10-hepatadecatrienoic acid (HHT).

lipids changes induced by thrombin. Besides confirming the higher potency of propentofylline versus pentoxyfylline, these data also indicated that PC hydrolysis was more sensitive to inhibition by methylxanthines than PI hydrolysis.

cAMP levels in platelets

Figure 4 reports the changes of cAMP levels in platelets measured under the same experimental conditions as those used in AA metabolism studies. In the presence of thrombin alone, some decrease in

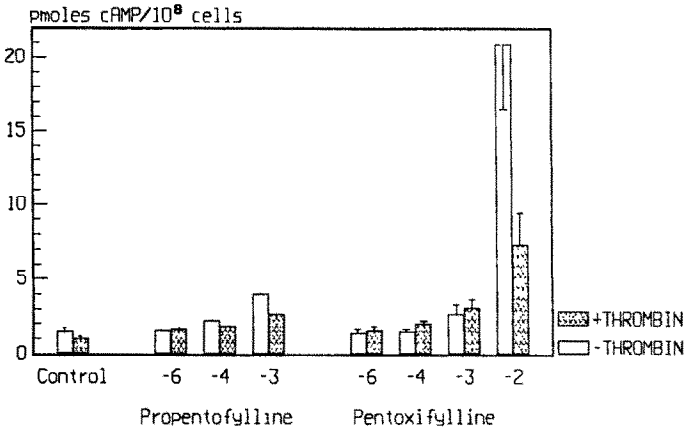

Fig. 4. Effects of pentoxifylline and propentofylline on human platelet cAMP levels in the absence (open bars) and in the presence (dotted bars) of thrombin (1 unit/ml). Results are means of 2 experiments for propentofylline and are means ± SEM of 3 experiments for pentoxifylline.

Table 5. Potentiation by propentofylline of prostaglandin E_1 inhibitory effect against metabolic changes induced by thrombin

	Prostaglandin E_1 (a)	Propentofylline (b)	Propentofylline + prostaglandin E_1 (c)	P (c/a)	P (c/b)
Phosphatidic acid	26.2 ± 7.1	9.8 ± 4.8	56.0 ± 4.6	<0.05	<0.01
Thromboxane B_2	41.8 ± 4.2	6.0 ± 2.3	74.0 ± 0.7	<0.01	<0.001
H.H.T.	45.1 ± 5.1	5.7 ± 8.4	77.7 ± 3.4	<0.01	<0.001
12-HETE	13.6 ± 2.0	−7.4 ± 9.0	57.8 ± 12.4	<0.05	<0.02

Propentofylline was preincubated 10 min with radiolabelled platelet suspension.
Prostaglandin E_1 was then added 5 min before addition of thrombin (1 U/ml).
Prostaglandin concentrations (75–300 nM) were chosen in order to produce around 25% of inhibition of phosphatidic acid production in each experiment. Propentofylline concentration was 10^{-5} M.
Results are means ± SEM of 3 separate experiments using platelets from different donors and are expressed as percentages of inhibition of the thrombin effect.
P, probability of significance according to Student's test.

cAMP content was found. Propentofylline and pentoxifylline clearly increase the cyclic nucleotide concentration at the highest concentrations used, 10^{-3} M in the case of propentofylline and 10^{-3} M–10^{-2} M in the case of pentoxifylline.

Synergistic effect of methylxanthines and prostaglandin E_1

A series of experiments was then undertaken in order to investigate possible synergistic effects of methylxanthines and prostaglandin E_1. As shown in Table 5, 10^{-5} M propentofylline, which did not significantly modify phosphatidic acid and icosanoid synthesis, increased the inhibition brought about by prostaglandin E_1 alone by 1.7 to 4 times, depending on the metabolite considered. Very similar data were also obtained in a typical experiment using 10^{-4} M pentoxifylline coupled to prostaglandin E_1 (not shown).

DISCUSSION

The first aim of our study was to define the interaction of pentoxifylline and propentofylline with platelets. Indeed, if *in vivo* beneficial effects of pentoxifylline have been reported in improvement of local haemostasis, beside their effects on erythrocyte deformability [32], no studies have yet demonstrated a direct interaction of this drug with platelets or other cells [33]. Using a protocol which did not involve washing of platelets after the incubation with the tritiated drugs, it has been possible to evidence some binding of the drugs to platelets. This is proportional to their concentration in external medium. Taking 10 μm^3 as a mean platelet volume [34], it could be calculated that the intracellular concentration of pentoxifylline and propentofylline would be 1.3- and 1.7-fold, respectively, that present in the extracellular medium (if considering a homogenous distribution of the drugs all over the aqueous intracellular compartment). Since these numbers are simply rough estimations, they must be considered with caution. At least, they indicate that the drugs equilibrate between intracellular and extracellular media probably by diffusion through the plasma membrane. This would explain facilitated access of these inhibitors to the cytosolic cAMP phosphodiesterase (see below). However, the slight apparent intracellular accumulation (1.3- and 1.7-fold) estimated by calculation might also indicate some accumulation of the drugs in some subcellular compartment, such as the lipid phase of the membrane. In this respect, the most lipophilic propentofylline displays a slightly higher enrichment. But in any case, such an interaction, which cannot be excluded, occurs very loosely, since both drugs are immediately removed by washing.

Pharmacological concentrations of pentoxifylline in blood are about 10^{-6} M [35], so the concentration necessary in our study to obtain a significant inhibition of [^{3}H] serotonin release and of aggregation of human platelets, between 10^{-4} M and 10^{-3} M are too high to assert, from our results, that pentoxifylline can inhibit platelet aggregation *in vivo*. However, previous *in vivo* studies have shown that this is actually the case [2, 3]. Studying the mechanism of this inhibitory effect on platelet has been the aim of our work.

For that purpose, phospholipid metabolism has been studied in thrombin-stimulated platelets using [^{14}C]AA labelled cells. Such a procedure is useful to follow at the same time the activation of phospholipase C, as detected by the accumulation of [^{14}C]PA and the activation of phospholipase A_2, as indicated by the liberation of [^{14}C]AA and its conversion into cyclooxygenase and lipoxygenase metabolites. Although some AA can be released by platelet DG lipase [13], the decrease in [^{14}C]PL is thought to reflect mainly hydrolysis by phospholipase A_2, whereas [^{14}C]PI hydrolysis is mainly due to phospholipase C activation, the majority of [^{14}C]DG being converted into [^{14}C]PA. Under these conditions, both pentoxyfylline and propentofylline were found to inhibit [^{14}C]AA liberation, so impairing the production of [^{14}C]icosanoids, as well as [^{14}C]PA accumulation. In agreement with these data, [^{14}C]PL and [^{14}C]PI hydrolysis was also inhibited in a dose-dependent manner, suggesting that phospholipase A_2 as well as phospholipase C were sensitive to the two methylxanthines. These results offer a good explanation to the fact that the same concentrations of pentoxifylline (10^{-2} M) and propentofylline

(10^{-3} M) inhibiting platelet phospholipases also suppressed platelet aggregation (Fig. 1) and secretion (Table 1). However, some dissociation could be found in the inhibition of PC and PI hydrolysis, the former (reflecting phospholipase A_2 inhibition) displaying a higher sensitivity to methylxanthines than phospholipase C. Since platelet phospholipases A_2 and C have been found to display different sensitivity to cytoplasmic free calcium concentration [39, 40], it would be tempting to speculate that phospholipase inhibition actually reflected some effect of the drugs on the level of cytoplasmic free calcium. Such an effect would be expected from drugs able to raise cAMP via inhibition of phosphodiesterase [25, 36–38]. However, platelet phospholipase C was found to be activated by thrombin in the absence of any elevation of cytoplasmic free calcium concentration [39, 41], suggesting possible additional mechanism.

In order to better understand the mechanism of platelet phospholipase inhibition induced by methylxanthines, cAMP was determined in platelet suspension incubated with various drug concentrations.

Platelet cAMP was found to increase in the presence of drugs, mainly at the highest concentrations used which were also those producing the main inhibitory effects on phospholipid metabolism. This is in agreement with the synergistic effects observed in the presence of a direct activator of platelet adenylate cyclase such as prostaglandin E_1. As previously discussed for other phosphodiesterase inhibitors [25], this might suggest a higher potency of methylxanthines against *in vivo* platelet activation, under which conditions prostaglandin I_2 is produced by endothelial cells. However, we cannot exclude at this time that, besides their inhibition of phosphodiesterase resulting in cAMP elevation, some additional effects of the drugs, which were found to equilibrate rapidly with platelet intracellular compartment, could also be involved in the observed changes.

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