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REGULATION OF HUMAN PLATELET ACTIVATION—ANALYSIS OF CYCLOOXYGENASE AND CYCLIC AMP-DEPENDENT PATHWAYS

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Abstract—We have studied the regulation of human platelet activation by cyclic AMP (cAMP), and the cyclooxygenase products by examining the effect of prostacyclin (PGI_2) and indomethacin on platelet aggregation, release reaction and thromboxane B_2 (TxB_2) generation induced by the full dose range of common platelet agonists in both platelet-rich plasma and washed platelets. Platelet aggregation, [^{14}C]-5HT and TxB_2 release induced by "threshold" and "supramaximal" concentrations of ADP, adrenaline, platelet-activating factor (PAF) and U46619 were totally abolished by low concentrations of PGI_2 (3–6 nM). In contrast, platelet activation induced by submaximal concentrations of collagen, thrombin and the calcium ionophore A23187 was only partially inhibited by PGI_2 (3–3000 nM). PAF-induced release reaction like that induced by ADP and adrenaline was totally dependent on the cyclooxygenase products and aggregation, while U46619-induced release reaction was only partially dependent on aggregation and the cyclooxygenase products. While both PGI_2 (18–3000 nM) and indomethacin (10 μM) abolished collagen-induced aggregation and the aggregation-mediated release reaction, neither inhibitor significantly inhibited platelet adhesion or the adhesion-mediated release reaction. Maximal thrombin-induced aggregation and release reaction was also not significantly inhibited by PGI_2 (300 nM) or indomethacin (10 μM). Thromboxane (TxB_2) generation induced by sub-maximal to maximal concentrations of collagen, thrombin and A23187 was, although significantly inhibited, not abolished by PGI_2. These results demonstrate that (a) PAF is a "weak" agonist similar to ADP and adrenaline, (b) U46619 is an agonist intermediate between weak and strong which induces a release reaction that is only partially dependent on aggregation, but unlike the strong agonists, is totally susceptible to inhibition by PGI_2, (c) PGI_2 is an indirect inhibitor of phospholipase activation, which does not significantly inhibit non-aggregation-mediated arachidonate mobilization, induced by the strong agonists, and (d) the so-called *third* pathway in the collagen and thrombin-induced release reaction, which is insensitive to indomethacin, is also insensitive to elevators of cAMP such as PGI_2.

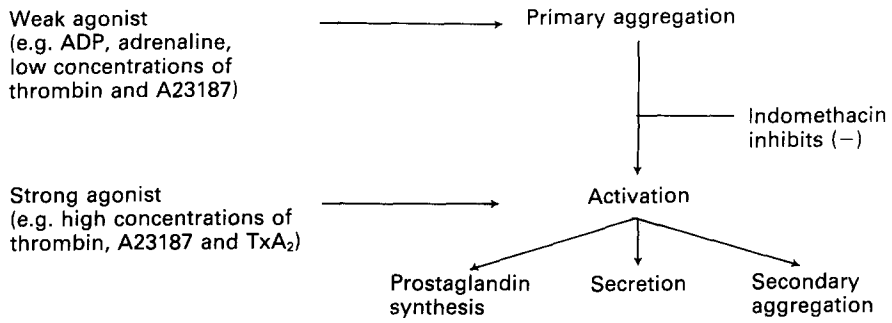
Detwiler and Huang [1] have proposed that platelet agonists are of two types—weak and strong. Their classification has been based on their findings, and those of others [2, 3], that dense-granule release induced by weak agonists such as ADP and adrenaline is inhibited by indomethacin, the cyclooxygenase inhibitor, and is dependent upon aggregation which requires stirring. The "release reaction" induced by strong agonists such as thrombin, the calcium ionophore A23187 and prostaglandin (PG)* endoperoxides/thromboxane (Tx) A_2, on the other hand, does not require stirring or aggregation, and is not inhibited by indomethacin. Thus, they have introduced the term "activation" which encompasses second-phase or secondary aggregation, prostaglandin synthesis and the release reaction, and used it to distinguish between direct activation caused by the strong agonists and aggregation-mediated activation which is caused by weak agonists (see Scheme I).

Unlike inhibitors of cyclooxygenase, agents that stimulate cyclic AMP (cAMP) synthesis such as PGE_1, PGD_2, PGI_2 [4–6], and adenosine are generally believed to be potent inhibitors [7, 8] of all the steps in the "basic platelet reaction" [9] both with weak and strong agonists. Inhibition of platelet phenomena by cAMP is thought to be due to a sequestration of cytoplasmic Ca^{2+} ions [10] which has been shown both by direct and indirect methods to play a central role in platelet activation [11–13].

However, the effect of cAMP on certain platelet phenomena such as the release reaction induced by thrombin and the calcium ionophore, A23187 [14–16] is still debatable. Although there is general agreement over the inhibition of platelet adhesion to subendothelium by PGI_2 [17, 18] studies on the effect of PGI_2 on platelet adhesion to collagen have also yielded conflicting results [19, 20].

The aim of this investigation has been to gain a better understanding of the regulation of human platelet activation by cAMP, and we have therefore examined the effect of PGI_2 on full dose–response curves of platelet agonists *in vitro*. Particular emphasis has been given to the effect of PGI_2 on collagen-induced platelet activation, and the distinction between aggregation and adhesion-mediated phenomena, platelet activation induced by thrombin, A23187, 15(*s*)hydroxy-11-9-epoxy-

* Abbreviations: cAMP, cyclic adenosine 3′,5′-monophosphate; PG, prostaglandin; Tx, thromboxane; PGI_2, prostacyclin; PRP, platelet-rich-plasma; WPL, washed platelets; LT, light transmission; 5-HT, 5-hydroxytryptamine.



Scheme I. Proposed by Detwiler and Huang [1] to contrast direct activation by strong agonists and aggregation-mediated activation by weak agonists.

methano prostadienoic acid (prostaglandin endoperoxide mimetic, U46619) [21] and 1-*O*-octadecyl-2-acetyl-*sn*-glyceryl-3-phosphoryl choline (platelet-activating factor or PAF) [22]. Throughout this study, the effects of PGI_2 have been compared to those of indomethacin to classify agonists by their susceptibility to cyclooxygenase inhibition or elevation of cAMP.

Platelet activation was measured as three parameters—aggregation, dense-granule release and thromboxane B_2 (TxB_2) generation in unstirred or stirred platelets. Collagen-induced adhesion and adhesion-mediated events were quantitated in unstirred platelets while aggregation and aggregation-mediated events were quantitated in collagen-stimulated stirred platelets.

MATERIALS AND METHODS

Materials. Adenosine diphosphate (ADP), epinephrine (adrenaline), indomethacin, creatine phosphate (CP), creatine phosphokinase (CPK) and collagen (Type I, insoluble) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). The collagen suspensions were prepared by the method of Packham *et al.* [23] and stored at 4°. Thrombin (bovine, Parke Davis & Co., Morris Plains, NJ) and Ca^{2+} ionophore A23187 (Calbiochem, Cambridge, U.K.), were made up in physiological saline and dimethyl sulphoxide (DMSO) respectively as stock solutions, and diluted on the day of the experiment to the required concentration. PAF was kindly supplied by Dr. R. Saunders (Sandoz, East Hanover, NJ). It was stored at −20° as a 10 mg/ml solution in a chloroform : methanol (4 : 1) mixture, and diluted in phosphate buffered saline (PBS, pH 7.4), containing 0.3% albumin to the required concentration. U46619, PGI_2 and TxB_2 were gifts from Dr. J. E. Pike (Upjohn Co., Kalamazoo, MI). U46619 and TxB_2 were made up as 1 mg/ml solutions in ethanol, stored at −20°, and diluted in Tris (0.1 M, pH 7.4) buffer when required. PGI_2 was stored solid and dry at −20°, and made up as a stock solution of 1 mg/ml in Tris (0.5 M, pH 10.5) buffer, which was stored at −20° as 10 μl aliquots. On the day of the experiment an aliquot was thawed, diluted in Tris (0.01 M, pH 10.5) buffer to the required concentration and stored on ice. [^{111}]-Indium chloride ($^{111}InCl_3$) (10 mCi/μg In) and [^{14}C]-5-hydroxy tryptamine creatinine sulphate ([^{14}C]-5HT, 30–50 mCi/mM) were obtained from Amersham, and [^{3}H]-TxB_2 (100–120 mCi/mM) from New England Nuclear (Southampton, U.K.).

Methods

Preparation of PRP, studies on platelet aggregation, dense-granule release from platelets prelabelled with [^{14}C]-5HT and radio-immunoassay of released TxB_2 were performed as previously described [24], except that collection of samples for [^{14}C]-5HT release was into a mixture of EDTA and formaldehyde [25] to prevent centrifugation-induced release of [^{14}C]-5HT.

Agonist-induced [^{14}C]-5HT or TxB_2 release in unstirred or stirred platelets, either in the presence of vehicle or inhibitor, was measured 180 sec after addition of agonist and calculated by subtracting non-specific release of [^{14}C]-5HT or TxB_2 obtained in the absence of agonist from that obtained upon agonist stimulation. Addition of vehicle or inhibitors was done 60 sec before addition of the agonist. Absence of aggregation in unstirred platelets was confirmed by phase-contrast microscopy. In experiments on the time-course of collagen-induced [^{14}C]-5HT or TxB_2 release, aliquots of PRP were removed 30, 60, 90 and 180 sec after collagen addition, and assayed as described before [24]. The cross-reactivity of the rabbit anti-TxB_2 antisera (kindly supplied by Dr. J. B. Smith, Philadelphia, PA) with U46619 was 0.017%, compared with 100% for TxB_2 under similar experimental conditions. The amount of TxB_2 produced by U46619-stimulated platelets was calculated by subtracting the apparent TxB_2 concentration due to cross-reactivity of the antisera with U46619 from the original value.

A range of agonist concentrations from "threshold" to "supramaximal" were tested against each inhibitor with "threshold" being the lowest concentration of agonist that produced greater than 5 pmoles of TxB_2 per 10^8 platelets, and underwent the release reaction, and "supramaximal" being the concentration two to five times the concentration that produced the maximal release reaction.

Preparation of washed platelets. Washed platelets (WPL) were prepared using a modification of the method of Ardlie *et al.* [26]. PRP was acidified with 0.1 N citric acid to pH 6.5 and EDTA added to a final

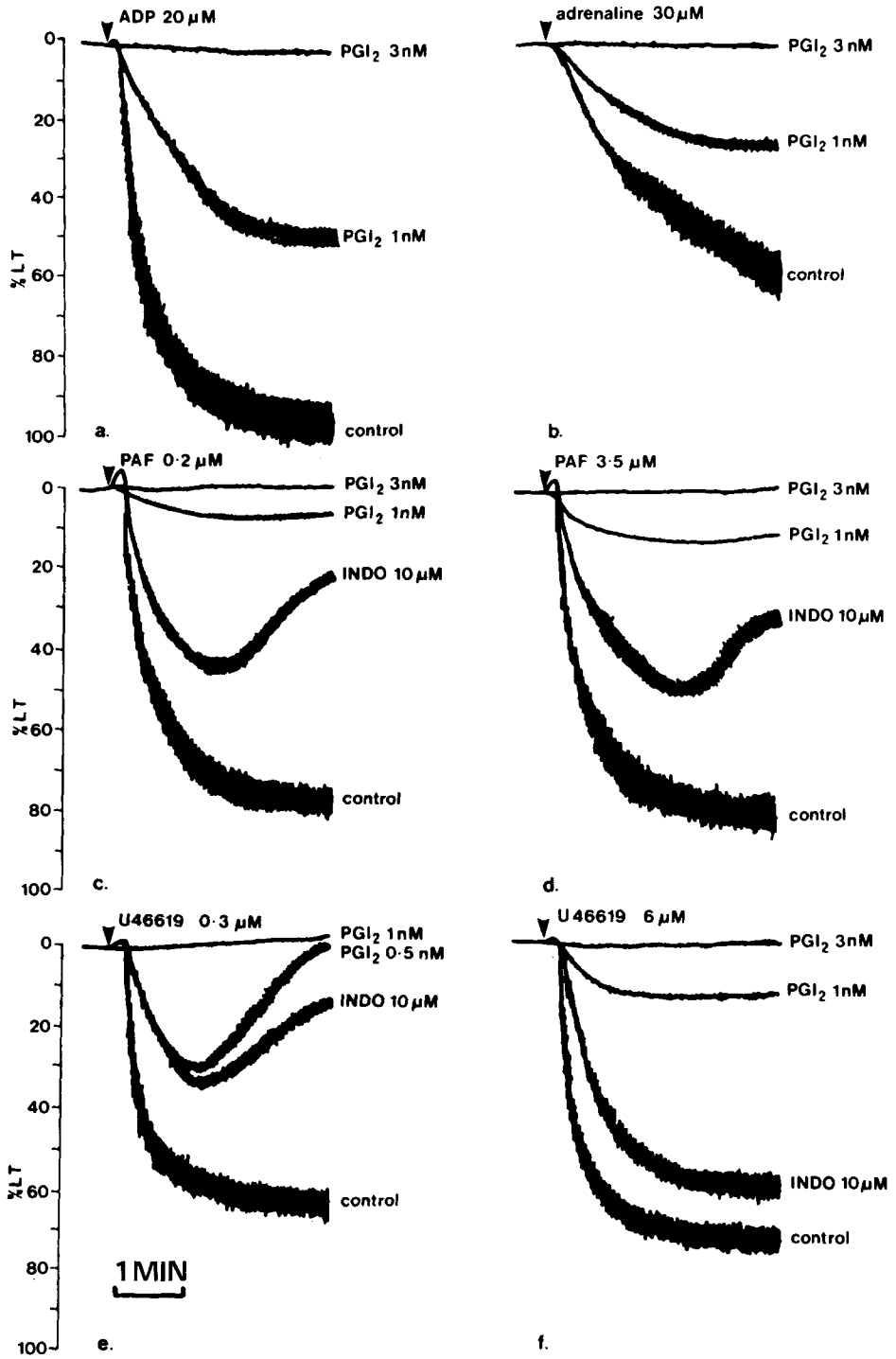


Fig. 1. Effect of PGI_2 and indomethacin on platelet aggregation induced by ADP (a), adrenaline (b), PAF (c and d) and U46619 (e and f). Aggregation was measured as the percentage increase in LT (vertical axis) with increasing time (horizontal axis) after addition of the agonist.

concentration of 1 mM. The PRP was centrifuged at 2500 *g* for 10 min, the platelet pellet washed twice in buffer (pH 6.5), containing citric acid (36 mM), potassium chloride (KCl, 5 mM), calcium chloride ($CaCl_2$, 1 mM), magnesium chloride ($MgCl_2$, 1 mM), sodium chloride (NaCl, 103 mM) and apyrase (75 μg/ml) (buffer 1). After two washes, the platelet pellet was resuspended in Tris–HCl (15 mM), KCl (5 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), NaCl (103 mM), at pH 7.4 (buffer 2). In experiments where glucose and albumin were added to the final resuspension buffer, the concentrations were 0.09 and 0.35% respectively.

Preparation of [^{111}In]-labelled platelets. Preparation of the [^{111}In]-oxine ([^{111}In]-hydroxyquino-

line) was by the method of Scheffel *et al.* [27]. Concentrated PRP (2 ml) was incubated with 6.25 μg of [^{111}In]-oxine for 30 min at 37°, which was then centrifuged at 2500 *g* for 10 min. The labelled platelet pellet was washed twice in buffer 1 and finally resuspended at a concentration of 10^9 platelets/ml in buffer 2 (pH 7.4), as described above. Glucose and albumin were omitted from the resuspension medium.

Measurement of platelet adhesion to collagen and the adhesion-mediated release reaction. The adhesion of platelets to collagen was measured using the method of Legrand *et al.* [28]. [^{111}In]-labelled platelets (400 μl containing 4×10^8 cells) were incubated with collagen (15 μg/ml) or vehicle for 180 sec at 37° without stirring, after which the platelets were filtered through Sepharose 2B columns (18 × 0.5 cm) at room temperature to separate adherent from non-adherent platelets. Elution of non-adherent platelets was done with Tris 0.1 M (pH 7.4) buffer. Ten fractions of 1 ml were collected from each column and the [^{111}In] in each fraction was measured in a Gamma counter. The percentage adhesion was calculated by expressing the difference between the radioactivity eluted in the presence and absence of collagen as a percentage of the radioactivity eluted in the absence of collagen. Where the effect of an inhibitor on adhesion was studied, the inhibitor or vehicle was added to the platelets 60 sec before collagen addition. The concentration of platelets and that of collagen were chosen so as to produce >70% adhesion, and absence of aggregation in these platelets was confirmed by phase-contrast microscopy.

Measurement of adhesion-mediated [^{14}C]-5HT release was carried out in WPL prepared in the same manner as for the adhesion assay, but labelled in PRP with [^{14}C]-5HT (0.75 μCi/10 ml PRP). [^{14}C]-5HT labelled WPL (400 μl containing 4×10^8 cells) were incubated with collagen (15 μg/ml) for 180 sec without stirring at 37°, and the reaction terminated with ice cold EDTA + formaldehyde. The incubates were then rapidly centrifuged in an Eppendorf centrifuge for 3 min and the [^{14}C] in the supernatant measured using standard liquid scintillation techniques.

Measurement of lactate dehydrogenase activity as an indicator of platelet lysis. Lactate dehydrogenase (LDH) activity in the platelet supernatants was quantitated by the measurement of NAD produced in the reduction of pyruvate to lactate as described by Lowry *et al.* [29].

Statistical analysis was performed using the Student's *t*-test for unpaired data.

RESULTS

The aggregation traces shown in Figs. 1, 2 and 4 are representative ones from 4–8 experiments, but the percentage [^{14}C]-5HT release and the pmoles of TxB_2 per 10^8 platelets shown in the tables are means ± S.E. of a total of at least eight determinations from different experiments.

Effect of PGI_2 on platelet activation induced by ADP, adrenaline, PAF and U46619 in PRP

Indomethacin abolished secondary aggregation, TxB_2 generation and [^{14}C]-5HT release induced by threshold (results not shown), and supramaximal concentrations of ADP (20 μM) and adrenaline (30 μM) (Table 1). It was found that concentrations of PGI_2 as low as 1–3 nM totally inhibited aggregation, TxB_2 and [^{14}C]-5HT release induced by both threshold (results not shown) and supramaximal concentrations of ADP and adrenaline (Figs. 1a and b, Table 1).

Table 1. Effect of PGI_2 and indomethacin on [^{14}C]-5HT release and TxB_2 generation induced by ADP, adrenaline, PAF and U46619

Agonist	Inhibitor*	[^{14}C]-5HT release (%)	P	TxB_2 (pmoles/10^8 pl)	P
20 μM ADP	None	56 ± 5.5		7 ± 3.5	
	PGI_2	0	<0.001	0.5 ±	<0.001
	Indomethacin	1.5 ± 0.8	<0.001	0	<0.001
30 μM Adrenaline	None	37.5 ± 2.4		26 ± 6.5	
	PGI_2	0	<0.001	1.5 ± 1.2	<0.001
	Indomethacin	0.8 ± 0.4	<0.001	0	<0.001
0.2 μM PAF	None	19.7 ± 3.7		10.5 ± 2.6	
	PGI_2	0	<0.001		<0.001
	Indomethacin	0	<0.001	0	<0.001
3.5 μM PAF	None	44.5 ± 4.5		22.5 ± 3.7	
	PGI_2	0.8 ± 0.2	<0.001	0	<0.001
	Indomethacin	0.2 ± 0.1	<0.001	0	<0.001
0.3 μM U46619	None	7.5 ± 2.5		7.5 ± 1.5	
	PGI_2	0	<0.001	0	<0.001
	Indomethacin	3.4 ± 0.1	<0.001	0	<0.001
6 μM U46619	None	23 ± 3.5		17.5 ± 2.5	
	PGI_2	0.5 ± 0.1	<0.001	0	<0.001
	Indomethacin	11.8 ± 2.4	<0.001	0	<0.001

* Inhibitors were 3 nM PGI_2, 10 μM indomethacin or vehicle (none) which was 0.2% alcohol or Tris buffer to substitute for indomethacin and PGI_2 respectively. These were added 60 sec before the agonist.

[^{14}C]-5HT and TxB_2 release into the supernatant of stimulated platelets were measured 180 sec after addition of the agonist.

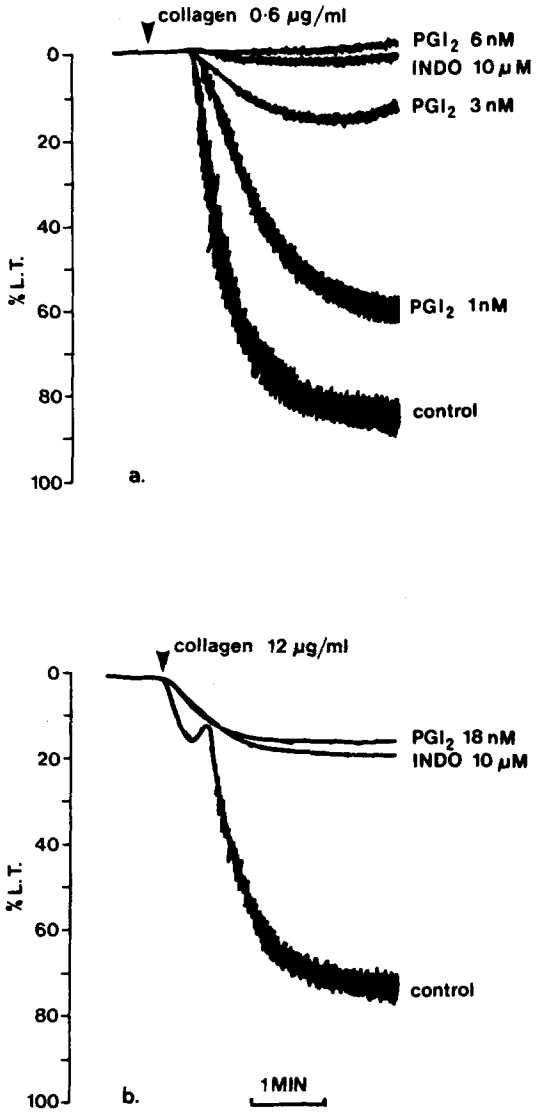


Fig. 2. Effect of PGI_2 and indomethacin on platelet aggregation induced by low (a) and high (b) concentrations of collagen.

Secondary aggregation, TxB_2 generation and [^{14}C]-5HT release at both threshold (0.2 μM) and supramaximal (3.5 μM) concentrations of PAF were inhibited by indomethacin (Figs. 1c and d, Table 1). Stirring was required for PAF-induced TxB_2 and [^{14}C]-5HT release. Incubation of unstirred platelets with 1–5 μM PAF produced no detectable [^{14}C]-5HT or TxB_2 release (data not shown). PGI_2 at concentrations of 1–3 nM totally inhibited threshold and supramaximal PAF-induced aggregation, TxB_2 and [^{14}C]-5HT release. PAF-induced shape change was also inhibited by PGI_2 (Figs. 1c and d).

Threshold and supramaximal U46619-induced [^{14}C]-5HT release was only partially inhibited by indomethacin (Table 1). Secondary but not primary aggregation at threshold concentrations of U46619 was inhibited by indomethacin although this inhibition was only sometimes present at higher concentrations of U46619 (Figs. 1e and f). At concentrations of 2 μM and above, U46619-induced [^{14}C]-5HT release occurred in unstirred platelets, although it was significantly ($P < 0.002$) lower than in stirred platelets, e.g. percentage [^{14}C]-5HT release induced by 6 μM U46619 was 11.5 ± 2.5% in unstirred platelets compared with 23 ± 3.5% in stirred platelets. U46619-induced TxB_2 generation, however, only occurred when the platelets were stirred, with no detectable TxB_2 in the supernatant of U46619 (1–6 μM)-stimulated unstirred platelets.

Threshold and supramaximal U46619-induced platelet activation, including shape change, was totally inhibited by 1–3 nM PGI_2 (Figs. 1e and f, Table 1).

Effect of PGI_2 on collagen-induced activation in stirred PRP

With collagen-induced platelet activation, two concentrations were used, a low concentration of 0.6 μg/ml where both the increase in light transmission (LT) and [^{14}C]-5HT release were totally inhibited by indomethacin and a higher concentration of 12 μg/ml, where partial inhibition of LT and [^{14}C]-5HT release by indomethacin were observed (Figs. 2a and b, Tables 2 and 3). PGI_2 at concentrations of 6–12 nM abolished low-dose collagen-induced aggregation, and the release reaction, although TxB_2 generation was only inhibited by 75% (Fig. 2a, Table 2).

Table 2. Effect of PGI_2 and indomethacin on low-dose collagen (0.6 μg/ml)-induced [^{14}C]-5HT release and TxB_2 generation

Inhibitor	%[^{14}C]-5HT release* (%)	P	TxB_2* (pmoles/ 10^8 pl)	P
None	35 ± 2		65 ± 5.4	
1 nM PGI_2	25.5 ± 3.2	<0.01	45 ± 5.6	<0.02
3 nM PGI_2	15 ± 4.5	<0.001	48 ± 4.8	<0.02
6 nM PGI_2	0.5 ± 0.2	<0.001	15.5 ± 5.7	<0.001
12 nM PGI_2	0	<0.001	16 ± 2.8	<0.001
3000 nM PGI_2	0	<0.001	11 ± 4.5	<0.001
10 μM Indomethacin	0	<0.001	0	

* Measured 180 sec after addition of collagen.

Table 3. Time course of high-dose collagen (12 μg/ml)-induced [^{14}C]-5HT and TxB_2 release

Inhibitor	30 sec	P	60 sec	120 sec	180 sec	P
			[^{14}C]-5HT release (%)			
None	10.7 ± 1.1		28.5 ± 0.7	44.4 ± 4.2	51 ± 3.5	
18 nM PGI_2	7.6 ± 2.6	N.S.			6.6 ± 0.6	<0.001
300 nM PGI_2	8.2 ± 1.5	N.S.			6.4 ± 0.6	<0.001
10 μM Indomethacin	7.9 ± 1.1	N.S.			7.5 ± 1.7	<0.001
10 μM Indomethacin + 4 mM CP + 4 U/ml CPK	8.4 ± 2.4	N.S.			8.5 ± 0.2	<0.001
			TxB_2 (pmoles/10^8 platelets)			
None	27 ± 3.5		55 ± 7.5	97 ± 5.5	11.5 ± 11	
18 nM PGI_2	16 ± 4.5	N.S.			53 ± 7.4	<0.001
300 nM PGI_2	14.2 ± 2.9	<0.01			48 ± 5.6	<0.001

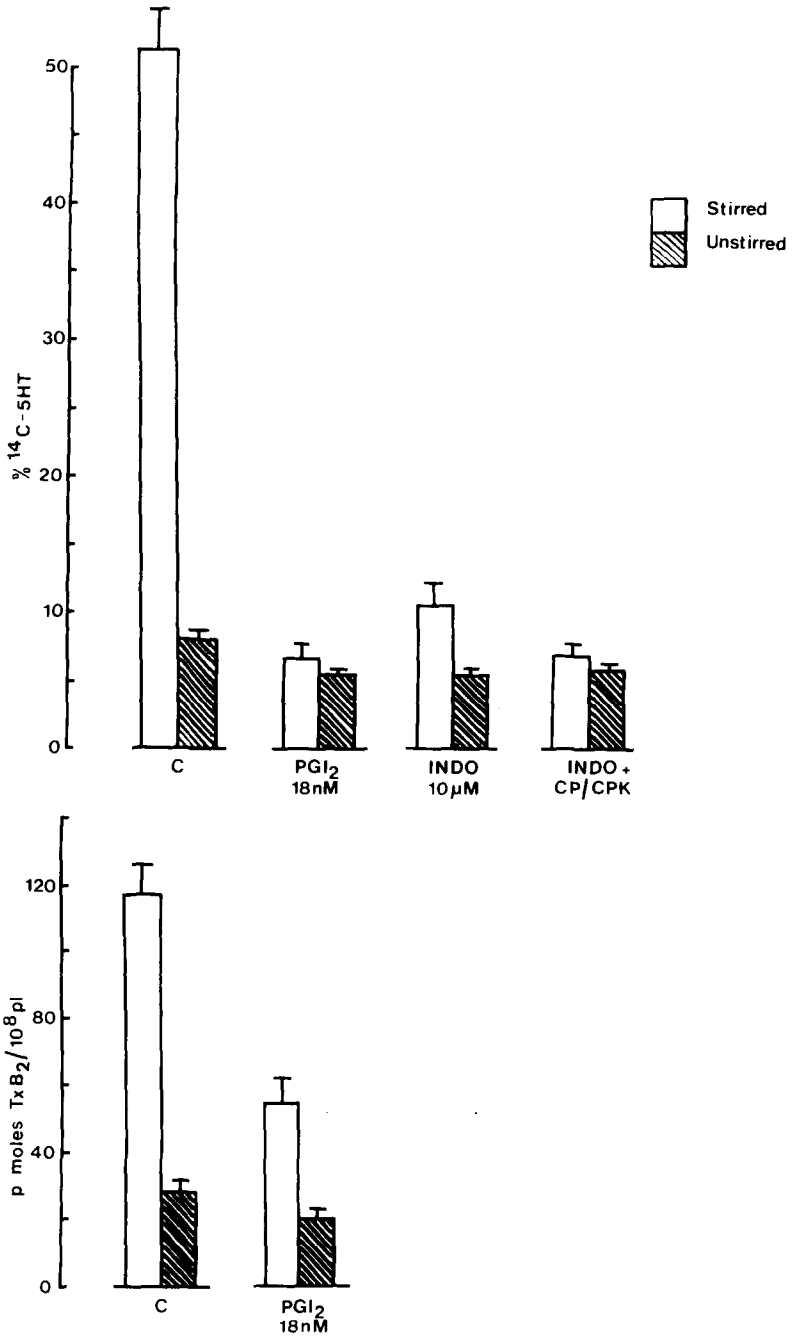


Fig. 3. Release of [^{14}C]-5HT and TxB_2 in collagen (12 μg/ml)-stimulated stirred and unstirred PRP measured 3 min after addition of collagen. 'C' represents the control response to collagen in the absence of drug.

Table 4. Effect of PGI_2 and indomethacin on platelet adhesion to collagen (15 μg/ml) and adhesion-mediated [^{14}C]-5HT release

Inhibitor	Adhesion* (%)	P	[^{14}C]-5HT release* (%)	P
None	77 ± 7.5		46 ± 6.4	
6 nM PGI_2	78 ± 4.7	N.S.	45 ± 5.8	N.S.
60 nM PGI_2	77 ± 5.5	N.S.	46 ± 3.5	N.S.
150 nM PGI_2	70 ± 6.4	N.S.	42 ± 2.5	N.S.
300 nM PGI_2	55 ± 6.6	<0.001	32 ± 3.7	<0.001
10 μM Indomethacin	79 ± 6.8	N.S.	47.5 ± 4.3	N.S.

* Measured in unstirred WPL 180 sec after addition of collagen.

At a concentration of 12 μg/ml, collagen-induced increase in LT was biphasic, consisting of an initial increase lasting for 15–30 sec which occurred immediately after the addition of collagen and a subsequent increase in LT which resembled the usual delayed aggregation response (Fig. 2b). The initial increase in LT, which was between 10 and 15%, was not inhibited by EDTA (5 mM) and probably represented the adhesion of platelets to collagen. Time-course studies on the release of [^{14}C]-5HT and TxB_2 upon the addition of collagen (12 μg/ml) revealed that the first-phase increase in LT was accompanied by small but significant amounts of [^{14}C]-5HT and TxB_2 release and was followed by much greater amounts of [^{14}C]-5HT and TxB_2 release accompanying the second-phase increase in LT (Table 3). Neither the first-phase increase in LT nor the accompanying [^{14}C]-5HT release was significantly ($P > 0.05$) inhibited by PGI_2 (3–3000 nM) or indomethacin, although both the second phase increase in LT and the accompanying [^{14}C]-5HT release were totally inhibited by PGI_2 (18 nM) and indomethacin (10 μM) (Fig. 2b, Table 3). Thus the release reaction after 180 sec in the presence of either inhibitor was not significantly different from that which occurred 30 sec after collagen addition, either in the presence or absence of inhibitor. Similarly, a mixture of indomethacin plus CP/CPK did not significantly inhibit the first phase collagen (12 μg/ml)-induced release reaction, although the second phase release reaction was totally blocked (Table 3).

First as well as second phase collagen (12 μg/ml)-induced TxB_2 generation was only partially inhibited by PGI_2 (3–3000 nM) (Table 3).

Effect of PGI_2 on collagen-induced activation in unstirred PRP

Unstirred platelets treated with collagen did not aggregate but adhered to the collagen fibrils as confirmed by phase-contrast microscopy, and underwent a small and consistent release reaction, as well as producing a significant amount of TxB_2, which was 15–25% of that occurring in stirred platelets. Collagen-induced release of [^{14}C]-5HT and TxB_2 in unstirred platelets was found with both low and high concentrations of collagen, although the amounts released were higher and more consistent with the higher concentration of collagen (12 μg/ml). Figure 3 summarizes the relation between the release of [^{14}C]-5HT and TxB_2 in collagen (12 μg/ml) stimulated stirred and unstirred platelets. The percentage [^{14}C]-5HT release 180 sec after collagen stimulation in unstirred platelets was not significantly ($P > 0.05$) different from the "first phase" release reaction (10.7 ± 1.1%) in stirred platelets (Table 3). This release reaction in unstirred PRP was not significantly ($P > 0.05$) inhibited by PGI_2 (18–3000 nM), indomethacin or indomethacin plus CP/CPK (Fig. 3). Collagen-induced TxB_2 generation in unstirred PRP was also not significantly ($P > 0.05$) inhibited by PGI_2 (18–3000 nM) (Fig. 3).

Effect of PGI_2 on platelet adhesion to collagen and adhesion-mediated [^{14}C]-5HT release in unstirred WPL

At concentrations of 6–150 nM, PGI_2 did not significantly ($P > 0.05$) inhibit platelet adhesion to collagen or the adhesion-mediated release of [^{14}C]-5HT (Table 4). At a concentration of 300 nM of PGI_2, a 30% inhibition ($P < 0.001$) of adhesion and [^{14}C]-5HT release was observed (Table 4). Concentrations of PGI_2 greater than 300 nM were not used because at these concentrations the drug caused >10% platelet lysis in WPL.

Neither adhesion nor the release of [^{14}C]-5HT was significantly ($P > 0.05$) affected by indomethacin (Table 4).

Effect of PGI_2 on thrombin and A23187-induced activation in PRP

At concentrations of 6–20 nM, PGI_2 totally inhibited threshold thrombin (0.2 U/ml)-induced aggregation, [^{14}C]-5HT release and TxB_2 generation (Table 5), although shape change remained unaffected (not shown). Inhibition of thrombin (0.2 U/ml)-induced TxB_2 generation by PGI_2 was partially overcome by an increase in thrombin concentration to 0.5 U/ml, which was a submaximal concentration, but was the highest concentration that could be used in PRP before clotting problems made it impossible to monitor the aggregation produced. At this concentration of thrombin, both aggregation and [^{14}C]-5HT release remained inhibited by 90% although TxB_2 generation was only inhibited by 60–65% by 20 nM PGI_2 (Table 5).

Platelet aggregation, [^{14}C]-5HT release and TxB_2 generation induced by threshold A23187 (4 μM) was totally inhibited by 6–12 nM PGI_2 (Table 5). Activation induced by 10–20 μM A23187 was maximal as measured by the release reaction, and at these

Table 5. Effect of PGI_2 on A23187 and thrombin-induced activation in PRP

Agonist	Inhibitor	LT* (%)	P	[^{14}C]-5 HT release* (%)	P	TxB_2* (pmoles/10^8 pl)	P
4 μM A23187	None	57 ± 6.6		67 ± 8.5		259 ± 94	
	6 nM PGI_2	0	<0.001	0	<0.001	5.4 ± 4.6	<0.001
20 μM A23187	None	90 ± 2.5		72.5 ± 7.2		712 ± 101	
	12 nM PGI_2	0	<0.001	5.9 ± 1.5	<0.001	167 ± 61	
	10 μM Indomethacin	87 ± 3.4	N.S.	68.5 ± 6.4	N.S.	not done	
0.2 U/ml Thrombin	None	82 ± 2.5		47.5 ± 4.8		26.5 ± 5.3	
	6 nM PGI_2	0	<0.001	4.5 ± 0.4	<0.001	0	<0.001
0.5 U/ml Thrombin	None	80 ± 4.5		58 ± 4.5		36 ± 7.4	
	20 nM PGI_2	5.5 ± 2.4	<0.001	2.6 ± 2.2	<0.001	29.5 ± 4.8	N.S.

* Measured 180 sec after addition of agonist.

concentrations of A23187, aggregation and [^{14}C]-5HT release, were inhibited by >90%, although TxB_2 generation was only inhibited by 60–70% (Table 5).

Neither aggregation nor [^{14}C]-5HT release induced by 20 μM A23187 was significantly inhibited by indomethacin (Table 5).

Effect of PGI_2 on thrombin-induced platelet activation in stirred and unstirred WPL

(a) *Stirred WPL.* Since concentrations of thrombin high enough to produce a maximal response could not be used in PRP, WPL resuspended in Ca^{2+}, Mg^{2+}, glucose and albumin containing buffer (pH 7.4) were used. Fibrinogen was omitted from the medium to avoid clotting. Low concentrations of PGI_2 (3–6 nM), totally inhibited threshold thrombin-induced (0.02 U/ml) aggregation, [^{14}C]-5HT and TxB_2 release (Fig. 4a, Table 6). Inhibition of thrombin (0.02 U/ml)-induced aggregation by 3–6 nM PGI_2 was easily overcome by increasing the thrombin concentration twofold to 0.04 U/ml, although inhibition was still observed with higher concentrations of PGI_2 between 15 and 20 nM (Fig. 4b).

At concentrations of 0.1–0.2 U/ml, activation of platelets by thrombin as measured by the [^{14}C]-5HT release was near maximal, and at these concentrations of thrombin, PGI_2 (18–300 nM) did not significantly ($P > 0.05$) affect the extent of aggregation, although the rate of aggregation was significantly ($P < 0.0001$) reduced by 100–300 nM PGI_2, e.g. 4.7 ± 0.5 to 0.8 ± 0.1 cm/min, with 300 nM PGI_2 (Fig. 4c). Thrombin-induced (0.1 U/ml) [^{14}C]-5HT release and TxB_2 generation was also not significantly ($P > 0.05$) inhibited by 6–150 nM PGI_2 although at higher concentrations of PGI_2 (150–300 nM), a 20–30% inhibition of [^{14}C]-5HT and TxB_2 release was observed (Table 6).

Indomethacin partially inhibited the aggregation and the release of [^{14}C]-5HT induced by 0.02 to 0.04 U/ml of thrombin, but did not significantly ($P > 0.05$) affect either the aggregation or [^{14}C]-5HT release induced by >0.04 U/ml of thrombin (Fig. 4, Table 6). TxB_2 generation induced by threshold and supramaximal concentrations of thrombin was totally inhibited by indomethacin (Table 6). Aggregation, but not [^{14}C]-5HT release, induced by all concentrations of thrombin was abolished by 5 mM EDTA (data not shown).

(b) *Unstirred WPL.* At concentrations of 0.05 U/ml and above, thrombin-induced release of [^{14}C]-5HT and TxB_2 occurred in unstirred platelets in the absence of aggregation. At concentrations of 3–150 nM, PGI_2 did not significantly ($P > 0.05$) inhibit the thrombin-induced release of [^{14}C]-5HT or TxB_2 in unstirred platelets, although a 15–30% inhibition was observed with 300 nM PGI_2. Thrombin-induced [^{14}C]-5HT release in unstirred WPL was also not significantly ($P > 0.05$) affected by indomethacin (Table 6).

DISCUSSION

Although there have been a number of studies performed on the effect of PGI_2 on platelet aggregation and the release reaction [6, 8, 30], a detailed

Table 6. Effect of PGI_2 and indomethacin on thrombin-induced [^{14}C]-5HT and TxB_2 release in WPL

Agonist	Inhibitor	[^{14}C]-5HT release* (%)	P	TxB_2* (pmoles/10^8 pl)	P
Stirred WPL					
0.02 U/ml thrombin	None	35.5 ± 7.2		1.8 ± 1.4	
	6 nM PGI_2	1.4 ± 0.8	<0.001	0	
	18 nM PGI_2	0		0	
	10 μM Indomethacin	17.7 ± 4.6	<0.01	0	
0.1 U/ml thrombin					
	None	76.5 ± 2.5		22.6 ± 4.2	
	6 nM PGI_2	78 ± 1.1	N.S.	27 ± 2.1	N.S.
	18 nM PGI_2	73 ± 2.4	N.S.	24 ± 3.2	N.S.
	150 nM PGI_2	77 ± 2.4	N.S.	25 ± 2.5	N.S.
	300 nM PGI_2	60 ± 1.5	<0.001	15 ± 4.2	<0.001
	10 μM Indomethacin	79 ± 2.8	N.S.	0	
Unstirred WPL					
0.1 U/ml thrombin					
	None	79 ± 1.1		18.6 ± 1.4	
	6 nM PGI_2	84.5 ± 2.2	N.S.	19.5 ± 1.1	N.S.
	150 nM PGI_2	78.5 ± 2.5	N.S.	15.4 ± 4.5	N.S.
	300 nM PGI_2	67 ± 1.5	<0.02	12.4 ± 1.5	<0.02
	10 μM Indomethacin	78.5 ± 1.2	N.S.		

* Measured 180 sec after addition of thrombin.

examination of the effect of PGI_2 on the three basic platelet phenomena, aggregation, release reaction and thromboxane generation, induced by different agonists under the same experimental conditions has been lacking. Furthermore, confusing data exist in the literature regarding the effect of PGI_2 on the thrombin-induced release reaction [14, 31, 32], platelet adhesion to collagen and collagen-induced platelet activation [19, 20]. One possible explanation for these conflicting reports could be the use of different concentrations of the agonists, and we have therefore examined platelet activation induced by a range of agonist concentrations from threshold to supramaximal, and the effect of PGI_2 on each agonist concentration. Such a detailed study has revealed firstly, that the discrepancies in the results of earlier workers was due to the use of single concentrations of agonists, and secondly, the existence of two distinct categories of agonists which were either completely susceptible (ADP, adrenaline, PAF and U46619) or not completely susceptible (collagen, thrombin and A23187) to inhibition by PGI_2. Thus maximal thrombin-induced activation as measured by the aggregation, [^{14}C]-5HT and TxB_2 release was only partially inhibited by PGI_2 (300 nM) in contrast to the results in earlier reports [14, 31, 32] where only single submaximal concentrations of thrombin were employed, and this finding could have important mechanistic and therapeutic implications. Similarly, previous studies [18–20] on collagen-induced platelet activation have tended to use single concentrations of collagen as well as to examine adhesion and aggregation in separate systems, and have not therefore been designed to make comparisons between the effect of inhibitors on aggregation and adhesion.

The use of stirred and unstirred platelets stimulated with low and high concentrations of collagen has enabled us to distinguish between aggregation-

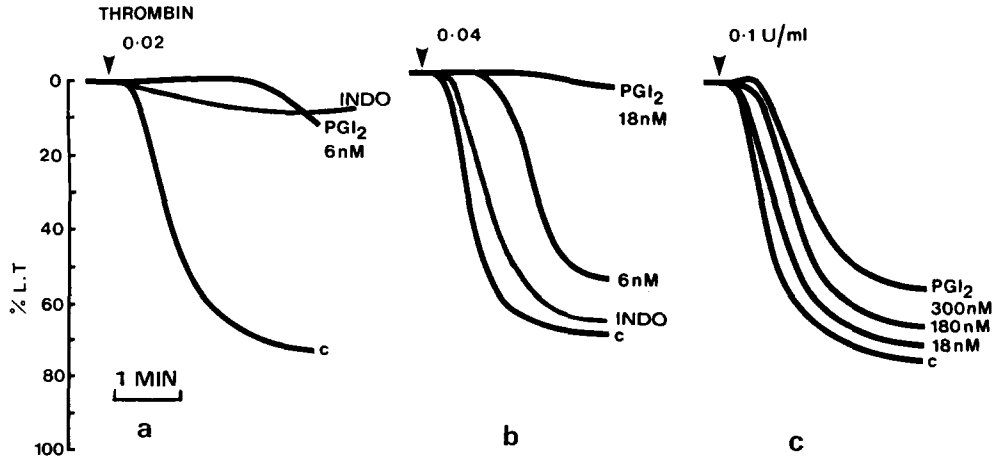


Fig. 4. Effect of PGI_2 and indomethacin on thrombin-induced aggregation of washed platelets.

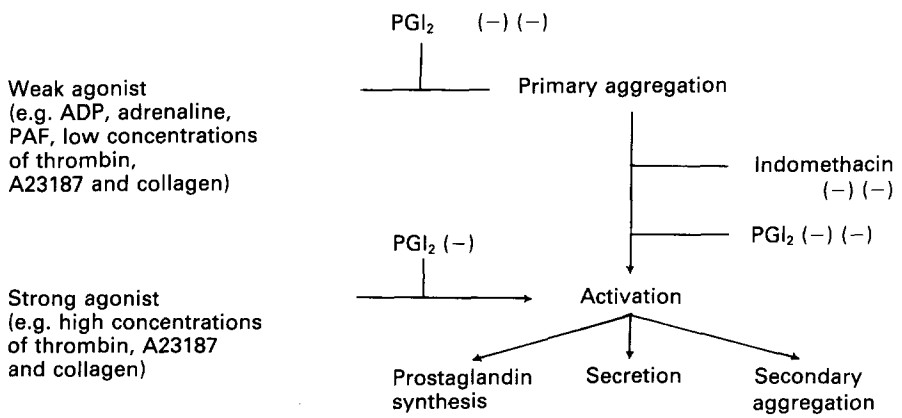
mediated and adhesion-dependent platelet activation and demonstrate that at concentrations that abolish collagen-induced aggregation neither PGI_2 nor indomethacin affect platelet adhesion to collagen or the adhesion-mediated release reaction. The significantly higher potency of PGI_2 at inhibiting platelet adhesion and the collagen-induced release reaction in the studies of Karniguian *et al.* [19] could also be due to the concentration of collagen used in their study and the possibility that the release reaction in their system was largely or completely aggregation-mediated rather than adhesion-mediated. Interestingly, our results agree with those of Cazenave and co-workers [20, 33], who used a different adhesion assay but reached similar conclusions.

The division of platelet agonists into two categories based on the effects of PGI_2 is broadly similar to the earlier classification [1] of agonists into weak and strong based on the effects of indomethacin, with the exception of the endoperoxide analogue, U46619. According to the earlier classification TxA_2 was classified as a strong agonist while in our study U46619 exhibited a similar susceptibility to PGI_2 as the weak agonists. It could be argued that although the stable PG endoperoxide/TxA_2 mimetic, U46619 induced a release reaction in unstirred platelets that was only partially inhibited by indomethacin this is not due to a mechanism independent of PG/Tx formation but due to the PG endoperoxide mimetic itself acting on the PG endoperoxide/TxA_2 receptor responsible for dense-granule release. Furthermore, since our studies revealed another difference between the actions of U46619 and the strong agonists in that U46619 induced no detectable TxB_2 generation in unstirred platelets it would seem reasonable to tentatively classify U46619 as an agonist that is "intermediate" between weak and strong until more information is available on the actions of PGG_2/H_2 or TxA_2 on platelets.

Examination of the effects of PAF, a proposed mediator of the "third" pathway in platelet activation [34, 35], has allowed us to classify it as a weak agonist with respect to inhibition by PGI_2 as well as indomethacin in human platelets. While this would gain support from the results of some earlier workers [36–38], it is at variance with that of others [39, 40]. It is unlikely that the "third" pathway in the thrombin and collagen-induced release reaction which is insensitive to both PGI_2 and indomethacin is due to PAF since PAF did not exhibit the characteristics required of a mediator capable of exerting the effects observed with collagen and thrombin.

With respect to the strong agonists, TxB_2 generation was less susceptible than aggregation or the release reaction to inhibition by PGI_2. This was apparent even at submaximal concentrations of collagen, thrombin and A23187 where, although aggregation and the release reaction were almost totally blocked by PGI_2, TxB_2 generation was only partially inhibited. In this context Feinstein and coworkers [41] working with PGE_1 and the local anaesthetics and their effect on A23187-induced phospholipase activation concluded that PGE_1 was an "indirect" inhibitor of phospholipase activation which acted by reducing cytoplasmic calcium and therefore its effect could be overcome by an increase in cytoplasmic calcium. Our results which demonstrate that PGI_2 is ineffective at inhibiting not only A23187-induced but also collagen- and thrombin-induced phospholipase activation suggest that PGI_2 is an "indirect" inhibitor which is ineffective at inhibiting aggregation-independent phospholipase activation induced by all strong agonists.

In conclusion, using our results on the effects of PGI_2 as well as indomethacin, we propose an extension of Detwiler and Huang's classification of weak and strong agonists (Scheme II). Weak agonists do not activate unstirred platelets and induce first primary aggregation, and then secondary aggregation, thromboxane generation and the release reaction, which are both cyclooxygenase and cAMP-dependent. Examples of weak agonists are ADP, adrenaline and PAF and low concentrations of thrombin, A23187 and collagen. Examples of strong agonists are thrombin, collagen and A23187 at high concentrations which induce platelet activation that is not dependent on aggregation, or cyclooxygenase activity, and only partially susceptible to intracellular



Scheme II. This Scheme of weak and strong agonists is a development of Scheme I [1] incorporating dependence on both cyclooxygenase products as well as intracellular cAMP levels. (−) (−), total inhibition; (−), partial inhibition.

cAMP levels. Consequently, these agonists are powerful inducers of thromboxane formation and dense-granule release in unstirred platelets. One agonist which was found to have characteristics distinct from the weak and strong agonists was the PG endoperoxide analogue, U46619, and further studies possibly using TxA_2 and other platelet agonists are required before U46619 can be included in the classification.

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