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Platelet desensitization induced by arachidonic acid is not due to cyclo-oxygenase inactivation and involves the endoperoxide receptor

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1 Human platelets pre-exposed to arachidonic acid (AA) (0.1–1 mM) or to the endoperoxide analogue U46619 (1–3 μM) and then washed and resuspended, failed to respond with aggregation or secretion to a second challenge by either agonist. The response to thrombin at low (0.04–0.1 u ml^{-1}) but not at high (2.5 u ml^{-1}) concentrations was also inhibited by pre-exposure to AA and U46619.

2 The ability of platelets to synthesize thromboxane (Tx) B_2 from AA or upon challenge with thrombin persisted despite platelet desensitization.

3 In the presence of the reversible cyclo-oxygenase (CO) inhibitors methyl salicylate (MS) or L8027, pre-exposure to AA had no effect on subsequent challenge by the same agonist or by U46619, whereas platelet desensitization by pre-exposure to U46619 persisted. However, platelet activation by, and desensitization to AA and U46619, was prevented by trimetoquinol and compound L636499, two thromboxane/endoperoxide receptor antagonists.

4 In contrast to the CO inhibitors, the thromboxane synthetase inhibitor dazoxiben, which in 3 'responders' out of 5 subjects suppressed aggregation, secretion, and Tx formation induced by AA, failed to prevent AA-induced desensitization.

5 Compared to quiescent cells the distances between platelets desensitized after re-exposure to AA were reduced in electron microscopy, but the tight connections associated with aggregated cells were not observed. Degranulation was also not observed and cell morphology resembled that of normal quiescent platelets.

6 In conclusion, (a) AA and U46619 desensitize human platelets at a similar site sensitive to prostaglandin/thromboxane receptor antagonists, and show cross-desensitization; (b) desensitization by AA appears to be mediated by a CO-dependent metabolite, as CO inhibitors prevent desensitization by AA but not to U46619; (c) the failure of dazoxiben to prevent desensitization by AA suggests that a metabolite other than TxA_2, possibly the endoperoxides, mediates the phenomenon; (d) desensitization does not involve inactivation of CO or thromboxane synthetase enzymes.

Introduction

Platelet activation by arachidonic acid (AA) (Vargaftig & Zirinis, 1973) is thought to be due to its transformation by cyclo-oxygenase (CO) into the prostaglandin endoperoxide PGG_2, which is readily reduced by peroxidase into PGH_2. Non-steroidal anti-inflammatory drugs (NSAID) block the transformation of AA into rabbit-aorta contracting substance (Vargaftig & Dao, 1971), now identified as thromboxane A_2 (TxA_2), a potent platelet aggregating agent formed from PGH_2 (Hamberg *et al.*, 1975). Even though TXA_2 synthesis is prevented by drugs such as imidazole (Needleman *et al.*, 1977) or dazoxiben (Tyler *et al.*, 1981; Bertelé *et al.*, 1981), in many instances aggregation by AA is not inhibited (Needleman *et al.*, 1977; Bertelé *et al.*, 1981). This suggests that the endoperoxides, which accumulate when thromboxane synthetase is blocked (Nijkamp *et al.*, 1977), induce aggregation *per se* (Bertelé *et al.*, 1981)

We have shown that pre-exposure of rabbit platelets

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to AA results in the subsequent desensitization of aggregation and secretion responses to this agonist, and that this can be prevented if the initial exposure is performed in the presence of reversible anti-CO agents (Rotilio *et al.*, 1984). Since the formation of the prostaglandin endoperoxides is initiated by a free radical chain reaction (Smith & Lands, 1972; Egan *et al.*, 1976; Hemler & Lands, 1980), which is suppressed when CO is inhibited by the NSAID, it was suggested that AA-derived peroxides are involved in CO inactivation in seminal vesicles (Egan *et al.*, 1976), which might be valid for platelets as well. An alternative possibility to explain platelet desensitization to AA which remains unexplored is that desensitization might occur at the level of the endoperoxide/thromboxane receptors. In the present study we examine this hypothesis, using human platelets exposed to AA or to the stable endoperoxide analogue U46619. Our results support the concept that desensitization to AA is independent of platelet secretion and involves the interaction of the endoperoxide/thromboxane agonists at a site distinct from CO and thromboxane synthetase.

Preparation of washed human platelets

Venous blood, provided by the Centre National de Transfusion Sanguine (Paris) was collected into heparin (10 u ml^{-1}) and citric acid-citrate-dextrose (7 mM, 93 mM and 139 mM, respectively; pH 6.5) from volunteers who denied drug consumption for at least 10 days before venipuncture. Platelet-rich plasma (PRP) was prepared by centrifugation (200 *g*; 20 min) at room temperature. Further centrifugation (1700 *g*; 15 min) yielded a platelet pellet which was then carefully broken down with a Pasteur pipette in modified Tyrode buffer according to Mustard *et al.*, (1972), in which apyrase was omitted. Platelets were washed three times by this procedure and the final resuspension adjusted to 500,000 platelet per μl.

Platelet aggregation and desensitization ('First phase experiments')

Washed platelets (4 ml) were transferred to 10 ml plastic tubes containing a stirring bar and kept at 37°C. Samples were pre-incubated with antagonists for 2 min before the addition of AA (0.1–1 mM) or U46619 (1–3 μM). Aggregation was evaluated by counting the number of free platelets detected at intervals with a Coulter Counter ZB1 (as shown in Figure 1). ATP secretion was measured by the luciferine-luciferase technique using an automatic dosing device ('Pico-ATP', Jobin Yvon, Paris, France). These determinations were performed before the addition of AA or U46619 and 1, 3 and 35 min after stimulation. Ten minutes later, PGI_2 (1 μM) was added for 25 min, to disaggregate the platelets. The platelets were then washed twice, centrifuged and the final resuspension in the modified Tyrode buffer adjusted to 500,000 per μl, before performing 'second phase experiments'. Control platelets (not exposed to AA or U46619, and otherwise run in parallel with the experimental samples, including the treatment with PGI_2) were recovered, and responded normally to the aggregating agents. The final recovery of control platelets in 7 experiments was 93 ± 2%, that of platelets exposed to 0.1 mM AA in 5 experiments was 95 ± 3%, and that of platelets exposed to 1 μM U46619 was 85 ± 4%.

Platelet aggregation ('Second phase experiments')

Approximately 1 h after platelet resuspension, aliquots (0.4 ml) of platelets which had been exposed to AA or to U46619, then washed, were transferred to aggregation tubes containing a stirring bar (1100 r.p.m.) and the fibrinogen content restored by the addition of DFP-fibrinogen (0.28 mg ml^{-1}). Platelets were then challenged at 37°C by AA (0.1 or 0.3 mM), U46619 (1 or 3 μM) or thrombin (0.04–0.1 u ml^{-1}, as a low concentration, or 2.5 u ml^{-1} as a high concentration). Second phase experiments were performed with a Chrono-log aggregometer and lasted approximately 3 h.

Radioimmunoassay for thromboxane B_2 determination

TxB_2 was assayed according to the method of Sors *et al.*, (1978). Briefly, aliquots (100 μl) of the platelet suspension were collected from the first or the second phase experiments 5 min after adding AA or U46619, left at room temperature for further 20 min and then stored at − 20°C. For performing the RIA the samples were thawed, and incubated overnight at 4°C with iodine-labelled TxB_2 and anti-TxB_2 serum in a γ-globulin buffer. The next day, bound and free fractions were separated by polyethyleneglycol 6000 precipitation, followed by centrifrugation at 2000 *g* and 4°C for 10 min. The radioactivity of the pellet, corresponding to the bound fraction, was counted for 1 min with an 'Intertechnique' gamma counter, directly connected to a Hewlett-Packard calculator.

Transmission electron microscopy studies

Platelets exposed to AA during first or second phase experiments were recovered after centrifugation and the resulting pellet was fixed and included, as described by Reynolds (1963). Slices (30–50 nm) were obtained using an ultramicrotome LKB III, and examined by a Siemens electron microscope.

Data presentation

The data are expressed as means ± standard error of the mean (s.e.mean). Statistical differences between two means were determined by Student's *t* test.

Materials

Compound U46619 15(S)-hydroxy-11,9(epoxymethano) prosta-5Z,13E-dienoic acid, was a gift from Dr J. Pike (Upjohn, Kalamzoo, U.S.A.). Arachidonic acid, bovine gamma globulin (Cohn fraction II), diisopropyl fluorophosphate (DFP) and methyl salicylate (MS) were from Sigma (U.S.A). Prostacyclin (PGI_2), provided by Dr S. Moncada (The Wellcome Research Laboratories, Beckenham, Kent) was dissolved in 0.05 M Tris buffer at pH 8.5 and stored at − 20°C. Methyl salicylate and compound L8027 (1'-(*iso*-propyl-2-indolyl) -3-pyrydyl-3-ketone from Sanofi, Toulouse, France) were dissolved in polyethylene glycol 300. Compound L636499 (dibenzo (b,f) thiepin-3-carboxylic acid,5,5 dioxide, a gift from Dr A. Ford-Hutchinson, Merck-Frosst, Quebec-Laval, Canada) was dissolved in 40% polyethylene glycol v/v in distilled water. Bovine thrombin and trimetoquinol (TMQ,1-(3',4',5'-trimethoxybenzyl)-6,7-dihydroxy-1-,2,3,4-tetrahydroisoquinoline, the R(+)-isomer) were from Hoffmann-La Roche, Basel, Switzerland. TMQ was prepared just before use in a phosphate-NaCl buffer at pH 7.4 containing 0.05% (w/v) sodium metabisulphite and kept at 4°C. Dazoxiben (4-(2-(1H-imidazol-l-yl) ethoxy) benzoic acid hydrochloride; Pfizer, U.S.A.) was dissolved in saline at a concentration of 0.1 mM; sonication was necessary for complete dissolution. Heparin was from Choay (Paris, France). Fibrinogen (Grade L) from Kabi (Stockholm, Sweden) was treated with DFP by standard procedures to inactivate coagulant contaminants.

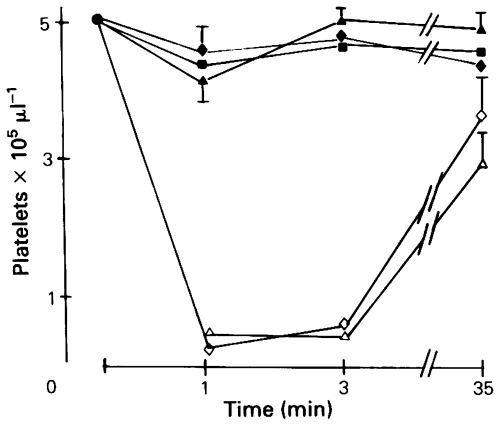


Figure 1 The effect of trimetoquinol (TMQ) on the reduction in the number of free platelets following stimulation with arachidonic acid (AA) or U46619 in 'first phase experiments'. The ordinate scale shows the number of free platelets per μl and the abscissa scale, time in min. TMQ (10 μM) was pre-incubated with platelets for 2 min before the addition of AA (0.1 mM) or U46619 (1 μM). Values are means ± s.e.mean for 4 experiments. (▲) TMQ plus U46619; (■) control; (◆) TMQ plus arachidonic acid; (◇) arachidonic acid; (△) U46619.

Results

Platelet activation induced by arachidonic acid or by U46619 ('First phase experiments')

Platelets stimulated by AA (0.1 or 0.3 mM) aggregated (Figure 1), secreted ATP and formed TxB_2 (Table 1). When AA was used at 1 mM the extent of aggregation and secretion was less than that obtained with lower concentrations (Table 1), possibly because of the formation of a larger amount of the anti-aggregatory

Table 1 Platelet activation by arachidonic acid (AA). Following stimulation by AA the number of free platelets per nl, the amount of ATP secreted (nmol ml^{-1}) at 1, 3 and 35 min and the amount of thromboxane B_2 (TxB_2) formed after 5 min are indicated (means ± s.e.mean for 3 experiments).

	1 min		*3 min*		*35 min*		
Arachidonic acid (mM)	*Number of free platelets per nl*	*ATP* (nmol ml^{-1})	*Number of free platelets per nl*	*ATP* (nmol ml^{-1})	*Number of free platelets per nl*	*ATP* (nmol ml^{-1})	*TxB_2* (ng ml^{-1})
Saline	423 ± 17	0.1 ± 0	423 ± 20	0.2 ± 0.1	443 ± 43	0.2 ± 0.1	23 ± 8
0.1	79 ± 28	4.1 ± 0.7	59 ± 13	7 ± 0.1	350 ± 63	6 ± 1	648 ± 49
0.3	82 ± 1	4.5 ± 0.5	34 ± 10	6 ± 0.2	310 ± 63	5 ± 0.2	1326 ± 351
1	227 ± 26	0.5 ± 0.2	332 ± 24	1 ± 0.1	356 ± 32	0.5 ± 0.3	1473 ± 35

Table 2 Release of ATP (nmol ml^{-1}) and formation of thromboxane B_2 (ng ml^{-1}) after platelet stimulation by arachidonic acid (AA, 0.1 mM) or U46619 (1 μM)

	ATP release (at 3 min) nmol ml^{-1}		*Thromboxane B_2* ng ml^{-1}	
Drug addition	*AA* (0.1 mM)	*U46619* (1 μM)	*AA* (0.1 mM)	*U46619* (1 μM)
No addition	8.6 ± 1.5	7.6 ± 1.5	423 ± 143	3.1 ± 1
MS 0.1 mM	0.8 ± 0.3*	4.6 ± 1.5	94 ± 22*	3.0 ± 1
L8027 0.1 mM	0.2 ± 0.0*	4.9 ± 1.7*	6 ± 4*	10.9 ± 9*
TMQ 0.01 mM	0.2 ± 0.0*	0.1 ± 0.0*	439 ± 61	12.5 ± 9
L636499 0.3 mM	0.2 ± 0.0*	0.1 ± 0.0*	67 ± 27*	18.9 ± 10*
Dazoxiben 0.1 mM	0.4 ± 0.0*	NT	4 ± 0.5*	NT

Prostaglandin/thromboxane antagonists or enzyme inhibitors at the concentrations indicated were applied as described in *Methods*.
NT not tested; MS methyl salicylate; TMQ trimetoquinol.
(1) stimulated with 0.3 mM AA; (2) stimulated with 3 μM U46619
*Significantly different ($P < 0.05$) when compared to the control untreated platelets stimulated with AA or U46619.

PGD_2, as occurs when thromboxane synthetase is inhibited by dazoxiben (Bertelé *et al.*, 1983).

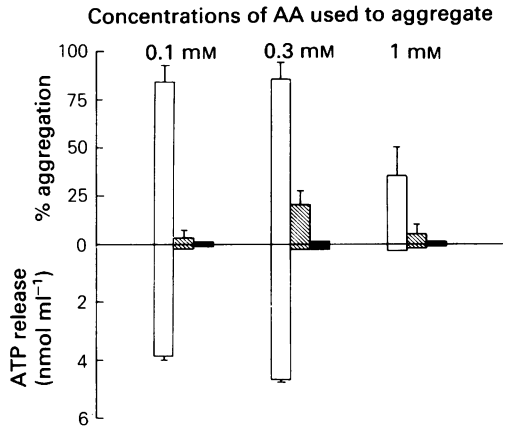
The endoperoxide analogue U46619 which has endoperoxide and thromboxane A_2-like effects (Armstrong *et al.*, 1983) at 1 μM induced marked aggregation (Figure 1) and secretion of ATP, comparable to that induced by AA (0.1 mM). In contrast to AA, which resulted in the generation of 423 ± 143 ng ml^{-1} of TxB_2, no significant formation of TxB_2 (3 ± 1 ng ml^{-1}) was obtained with U46619 (Table 2).

The reversible CO inhibitors MS and compound L8027 (0.1 mM for both), added to the platelet suspension 2 min before the agonist, inhibited secretion, formation of TxB_2 (Table 2) and aggregation (not shown) induced by AA (0.1 mM). Secretion by U46619 (1 μM) was reduced non significantly by MS or L8027 (Table 2). In contrast, the endoperoxide/ thromboxane receptor antagonists TMQ (10 μM) (MacIntyre & Willis., 1978; Mayo *et al.*, 1981; 1983) and L636499 (0.3 mM) (Carrier *et al.*, 1984; Chan *et al.*, 1984)

Table 3 Ability of platelets pre-exposed in the presence of antagonists to arachidonic acid (AA, 0.1 mM) or U46619 (1 μM) to release ATP when re-stimulated with AA, U46619 or thrombin at the doses indicated

	ATP-release nmol ml^{-1} (at 3 min) *Stimulating agent*		
Pretreatment	*AA* (0.1 mM)	*U46619 (1 μM)*	*Thrombin* (2.5 u ml^{-1})
Saline (no pretreatment)	6.8 ± 1.8	4.3 ± 1.4	8.7 ± 2.0
AA alone	0.1 ± 0.1*	0.1 ± 0.1*	2.6 ± 0.7*
MS ± AA	4.8 ± 1.1	3.1 ± 0.6	9.3 ± 1.9
L8027 + AA	4.1 ± 1.8	4.1 ± 1.6	9.1 ± 1.7
TMQ + AA	7.6 ± 2.2	5.0 ± 0.3	13.4 ± 1.7
L636499 + AA (1)	5.1 ± 2.3	4.3 ± 2.6	10.8 ± 1.9
Dazoxiben + AA	0.2 ± 0.1*	0.3 ± 0.2*	7.7 ± 2.9
U46619 alone	0.2 ± 0.1*	0.2 ± 0.1*	2.2 ± 0.6*
MS + U46619	1.5 ± 0.7*	0.6 ± 0.4*	5.4 ± 1.5
L8027 + U46619	0.2 ± 0.1*	0.1 ± 0.1*	2.8 ± 0.8*
L636499 + U46619	6.3 ± 1.9	3.9 ± 2.3	10.7 ± 1.9
TMQ + U 46619 (2)	7.5 ± 1.9	4.0 ± 0.6	12.3 ± 2.3

Antagonists used were methyl salicylate (MS; 0.1 mM), L8027 (0.1 mM), trimetoquinol (TMQ, 0.01 mM), L636499 (0.3 mM), or dazoxiben (0.01 mM). (means ± s.e.mean of 4 experiments).
(1) pre-exposed to 0.3 mM AA; (2) pre-exposed to 3 μM U46619.
*Significantly different ($P < 0.05$) when compared to the control non pre-treated platelets stimulated with AA, U46619 or thrombin.



suppressed the effects of both agonists (Figure 1 and Table 2). L636499 reduced the formation of TxB2 from AA (Table 2) as much as MS. Finally, the thromboxane synthetase inhibitor dazoxiben inhibited aggregation and secretion induced by AA in 3 out of 5 donors, despite the fact that synthesis of TxB_2 was suppressed in all 5 cases (Table 2).

Figure 2 Desensitization of platelets to arachidonic acid (AA). Platelets were exposed to saline (open columns) or to AA (0.1 mM, hatched columns; 0.3 and 1 mM, closed columns) during 'first phase experiments', then washed and recovered as described in Methods and stimulated with 0.1, 0.3 or 1 mM AA. Aggregation and ATP release were monitored as indices of functional responsiveness and are shown on the ordinate scale as a percentage of maximal response and in nmol ml^{-1}, respectively (means of 4 experiments; s.e.means indicated by vertical lines).

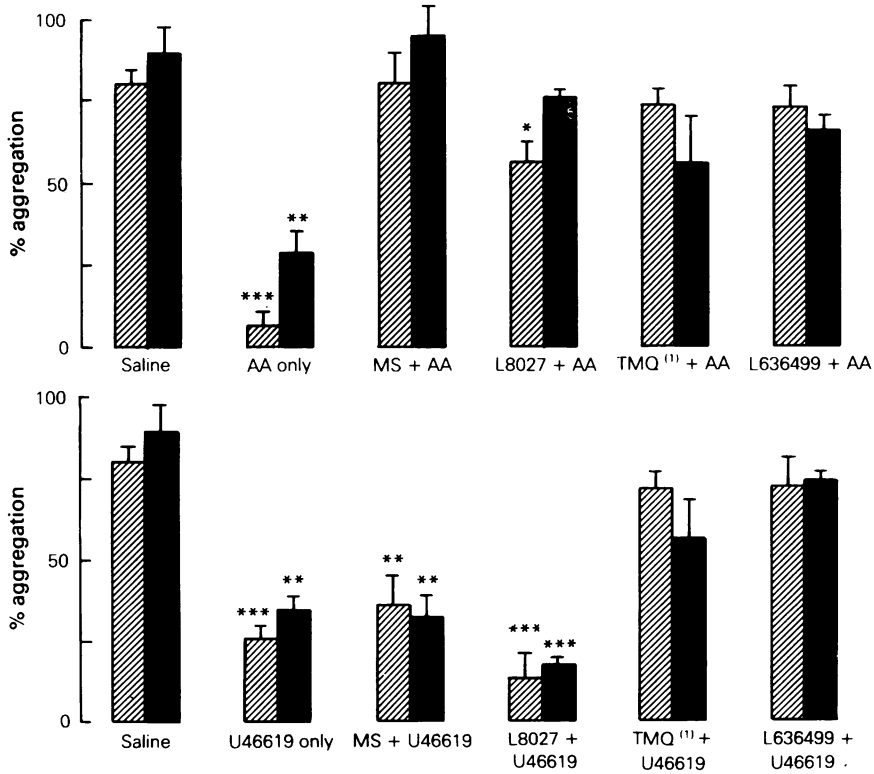


Figure 3 Drug modulation of arachidonic acid (AA) and U46619-induced platelet desentiziation. Methyl salicylate (MS, 0.1 mM), L8027 (0.1 mM), trimetoquinol (TMQ; 0.01 mM), L636499 (0.3 mM) or saline in controls were pre-incubated with platelets for 2 min before exposure to AA (0.1 mM; upper section) or U46619 (1 μM; lower section).

Platelets were then washed and recovered as described in Methods. Platelet aggregation induced by AA (0.1 mM; hatched columns) or U46619 (1 μM; solid columns) was monitored 3 min after agonist addition, as an index of functional response and is shown on the ordinate scale as a percentage of maximal response (means of 4 experiments; s.e.mean shown by vertical lines). Significance: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 4 Production of thromboxane B_2 (TxB_2) by platelets desensitized to arachidonic acid (AA, 0.1 mM) or U46619 (1 μM), in the presence of the prostaglandin/thromboxane antagonists at concentrations shown in Table 3, and re-stimulated with AA or with U46619, compared to aggregation by a low and by a high concentration of thrombin, at the indicated concentrations

	Stimulating Agent			
	Thrombin (2.5 μml^{-1})	*Thrombin* (0.04–0.1 μml^{-1})	*AA* (0.1 mM)	*U46619* (1 μM)
Pretreatment	*% aggregation*		*TxB_2 (ng ml^{-1})*	
Saline (no pretreatment)	63 ± 5	57 ± 4	215 ± 99	9 ± 8
AA alone	45 ± 11*	11 ± 6*	207 ± 125	19 ± 9
MS + AA	61 ± 6	74 ± 9	527 ± 280	26 ± 8
L8027 + AA	45 ± 1	47 ± 8	295 ± 184	2 ± 0.4
TMQ + AA (1)	50 ± 7	8 ± 4*	261 ± 18	19 ± 0.4
L636499 + AA	52 ± 4	52 ± 3	604 ± 133	42 ± 18
Dazoxiben + AA	48 ± 3	NT	123 ± 69	NT
U46619 alone	45 ± 14	8 ± 6*	230 ± 71	6 ± 4
MS + U46619	41 ± 5*	17 ± 10*	265 ± 81	17 ± 13
L8027 + U46619	38 ± 5*	7 ± 7*	206 ± 31	2 ± 0.4
TMQ + U46619(2)	52 ± 5	3 ± 2*	296 ± 16	6 ± 5
L636499 + U46619	55 ± 3	56 ± 3	571 ± 79	28 ± 3.3

NT not tested; MS methylsalicylate: TMQ trimetoquinol.
(1) pre-exposed to 0.3 mM AA; (2) pre-exposed to 3 μM U46619.
*Significantly different ($P < 0.05$) when compared to the control non pre-treated platelets stimulated with AA, U46619 or thrombin.

Platelet desensitization induced by arachidonic acid or by U46619 ('Second Phase experiments')

Control untreated platelets and platelets exposed to AA (0.1, 0.3 or 1 mM) or to U46619 (1 μM) were disaggregated with PGI_2 (1 μM); 25 min later, the platelets were washed to remove drugs. These platelets failed to aggregate or secrete when re-stimulated with AA (0.1–1 mM) (Figures 2 and 3; Table 3), U46619 (1–3 μM) (Figure 3; Table 3) or with low concentrations of thrombin (0.04–0.1 u ml) (Table 4). However, although platelets pre-exposed to AA or U46619 did not aggregate on subsequent challenge by either agonist, a distinct shape change could be observed

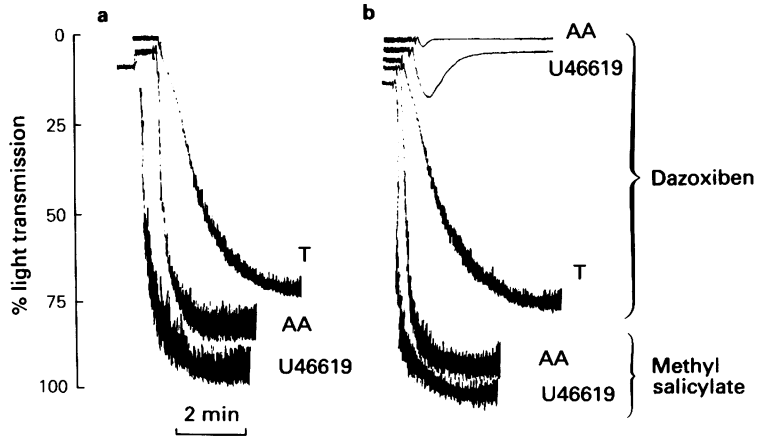


Figure 4 Platelet aggregation induced by arachidonic acid (AA, 0.1 mM), U46619 (1 μM) and thrombin (T, 2.5 u ml^{-1}) using platelets from a dazoxiben 'responder' (see text); effect of dazoxiben and methylsalicylate (MS). Platelets were pre-incubated with dazoxiben (0.01 mM) or MS (0.1 mM) for 2 min before exposure to AA (0.1 mM). After recovery platelet aggregation in these platelets (b) or in control platelets (no drug treatment, a), were monitored in response to stimulation by the agonists. Aggregation is shown as a percentage of maximal response.

(data not shown). These cells also aggregated in response to high concentrations of thrombin (2.5 u ml^{-1}) (Table 4).

Pre-exposure to low concentrations of AA (0.1 or 0.3 mM) did not alter the ability of the cells to generate TxB_2 on subsequent challenge with 0.1 and 0.3 mM AA, generation from 1 mM AA being half-inhibited (Figure 5). In this series of experiments, control platelets (not pre-exposed to AA) generated 197 ± 80 ng ml^{-1} TxB_2 when challenged with 0.3 mM AA; 736 ± 312 ng ml^{-1} when challenged with 0.3 mM AA and 1537 ± 447 ng ml^{-1} when challenged with 1 mM AA. Platelets pre-exposed to 0.1 mM AA and then stimulated, generated 251 ± 73 ng ml^{-1} TxB_2 from 0.1 mM AA, 866 ± 36 ng ml^{-1} from 0.3 mM AA and 1618 ± 448 ng ml^{-1} from 1 mM AA. Platelet exposure to 0.3 mM AA also failed to modify significantly the formation of TxB_2 from 0.1 or 0.3 mM AA. In contrast, platelet pre-exposure to 1 mM AA abolished TxB_2 formation on subsequent challenge with the three concentrations of AA (Figure 5) but also prevented aggregation induced by high concentrations of thrombin (2.5 u ml^{-1}) (data not shown).

Drug modulation of platelet desensitization by arachidonic acid or U46619

Pre-treatment of platelets with either the reversible CO inhibitors, MS or L8027, or the prostaglandin thromboxane receptor antagonists TMQ or L636499 in the first phase, prevented desensitization induced by AA. Hence, following stimulation by AA or U46619 in the second phase, these platelets aggregated and secreted normally (Figures 3 and 4; Table 3), although there was a small but significant reduction in aggregation of L8027-pretreated platelets (Figure 3). Unlike the prostaglandin/thromboxane receptor antagonists, the enzyme inhibitors (MS or L8027) were ineffective in preventing desensitization induced by U46619 (Figures 3 and 4; Table 3). In some instances, when TMQ was used to prevent desensitization induced by U46619 or AA, higher concentrations of these agonists (3 μM U46619 or 0.3 mM AA), which had no effect on desensitized platelets, were required to induce aggregation and secretion.

Dazoxiben inhibited AA-induced aggregation during the 'first phase experiments' in 3 out of 5 cases. This was not unexpected, since dazoxiben suppresses the formation of TxB_2 from AA by human platelets, but aggregation is only inhibited in approximately half the cases, which allows the distinction of dazoxiben 'responders' from 'non-responders' (Bertelé *et al.* 1981; 1983). Although dazoxiben prevented platelet aggregation and secretion induced by AA in the first phase (Table 2) when platelets from 'responders' were used, this agent did not protect the platelets from desensitization induced by AA or U46619 (Figure 4 and Table 3). That these platelets retained their granule contents was demonstrated by electron microscopy (see below). Moreover, when treated with the powerful secretagogue thrombin (2.5 u ml^{-1}) in the second phase, these platelets aggregated (not shown) and secreted ATP as much as control platelets (Table 3).

Electron microscopy

Platelets exposed to AA during the first phase were aggregated and degranulated (compare Figure 6b to control in Figure 6a). Degranulation and aggregation were not observed in platelets co-exposed to AA and dazoxiben (Figure 6c). In second phase experiments, non pre-exposed control platelets stimulated with AA were aggregated and degranulated (Figure 6d), whereas those exposed to AA during the first phase were degranulated, but failed to aggregate upon re-exposure to AA (Figure 6e). Finally, platelets co-exposed to AA and to dazoxiben and restimulated with AA were not degranulated (Figure 6f). Nevertheless, as shown in Figure 6f, the inter-platelet distances were reduced, but the tight connections observed in aggregates were not found (compare Figures 6b and 6d).

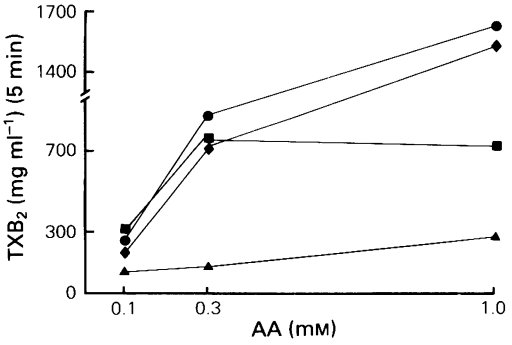


Figure 5 The effect of arachidonic acid (AA) induced desensitization on generation of platelet thromboxane B_2 (TxB_2). Values obtained in ng ml^{-1} given in the text; the s.e.mean are omitted from the figure for clarity. Control (◆); arachidonic acid 0.1 (●), 0.3 (■) and 1.0 (▲) mM.

Discussion

Arachidonic acid and the endoperoxide analogue U46619 both induced platelet aggregation and secretion of the granule constituent ATP (Figures 1, 2, 3 and 4; Tables 1, 2 and 3). These agents exhibited the phenomenon of homologous desensitization and also cross-desensitized with each other (Figures 2, 3 and 4). Aspirin produces an irreversible inhibition of CO (Roth & Majerus, 1975; Roth *et al.*, 1975). Since in our

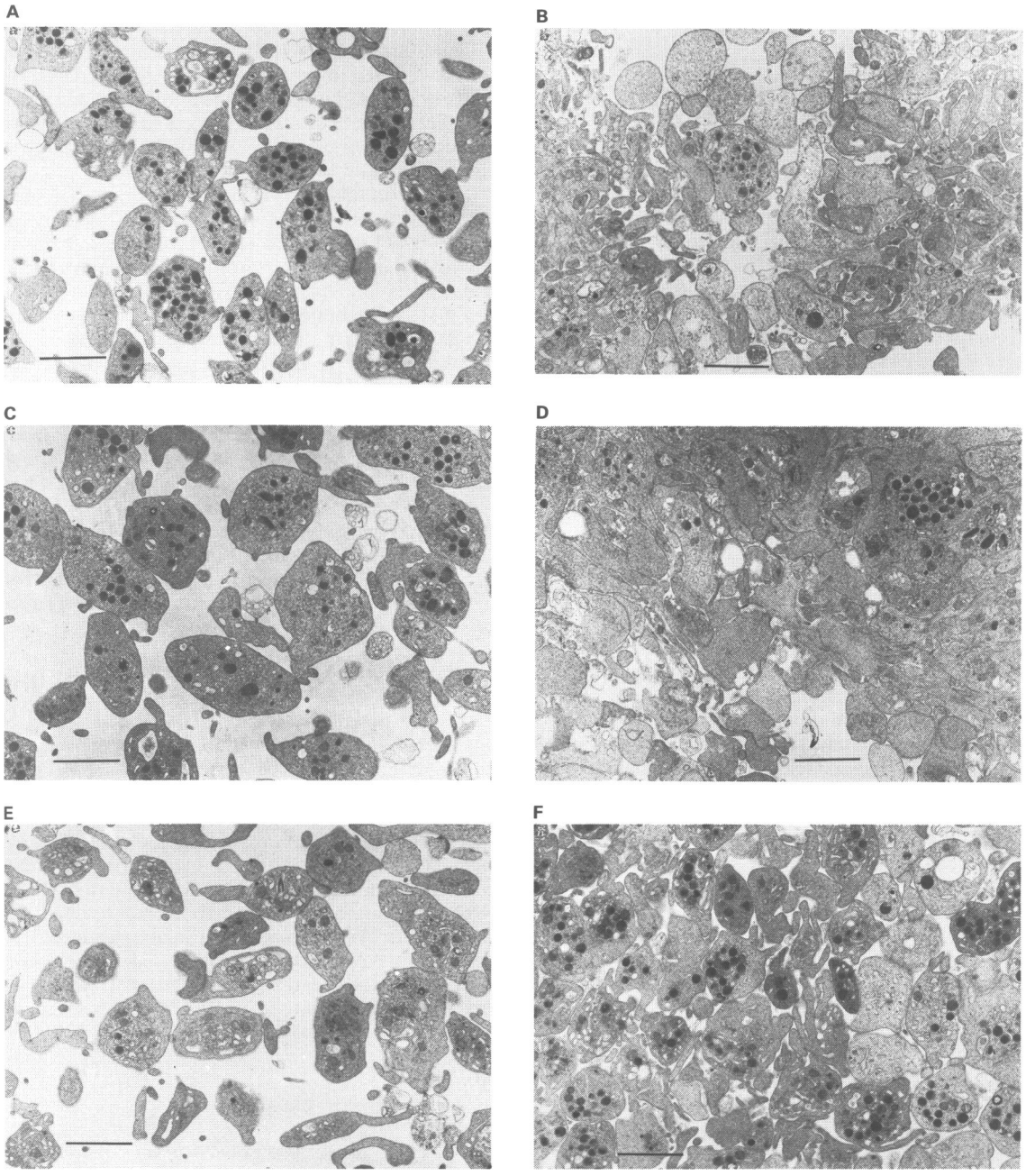


Figure 6 Electron micrographs of platelets collected after 3 min of stirring. Samples (a), (b) and (c) from first phase experiments: (a) control; (b) arachidonic acid (AA); (c) dazoxiben (0.01 mM) and arachidonic acid. Samples (d), (e) and (f) were collected during second phase experiments: (d) control platelets, as in (a), and re-exposed to AA; (e) platelets pre-exposed to AA, as in (b), and re-exposed to it; (f) platelets co-exposed to dazoxiben and to AA, as in (c), and re-exposed to AA. Concentrations of AA of 0.1 mM. Bars indicate 1.5 μM.

experiments platelets were to be recovered by washing, we used L8027 or MS, which inhibit CO in a reversible manner (Prancan *et al.*, 1979; Rotilio *et al.*, 1984), to modulate desensitization by AA. Both MS and L8027 and the thromboxane synthetase inhibitor dazoxiben (when platelets from 'responders', i.e., individuals whose platelets do not aggregate to AA in presence of dazoxiben, were used; see Bertelé *et al.*, 1981; 1983) prevented platelet activation induced by AA, but had no effect on activation induced by U46619. The prostaglandin/thromboxane antagonists TMQ (MacIntyre & Willis, 1978; Mayo *et al.*, 1981; 1983) and L636499 (Carrier *et al.*, 1984; Chan *et al.*, 1984) abolished the effects of both AA and U46619, indicating clearly that platelet activation during first phase experiments involves the interaction of U46619 with a common site. Compound L636499 also reduced the formation of TxB_2 from AA (Table 2), and cannot thus be classified as a strictly pure site antagonist. Its CO or thromboxane synthetase inhibitory activity nevertheless does not account for the antagonism by L636499 of U46619-induced platelet desensitization, which does not involve CO activation, as shown by the absence of synthesis of TxB_2 by U46619-stimulated platelets (Table 2). Furthermore, the levels of TxB_2 detected when platelets were co-exposed to AA and to L636499 were comparable to those detected when MS was used, indicating a similar extent of inhibition of the generation of TxB_2. Despite this similarity, MS did not interfere with platelet desensitization by U46619, whereas L636499 did. L636499 thus behaved as a mixed enzyme/site inhibitor, but its protective effect against platelet desensitization involves, at least in part its site-directed effects. In recent experiments (M. Hatmi, D. Joseph and B.B. Vargaftig, unpublished) human platelets exposed to the prostaglandin endoperoxide/thromboxane mimetic EP171 (Jones *et al.*, 1985) and recovered as here, underwent self-desensitization as well as cross-desensitization with AA and with U46619, confirming the concept of desensitization at the prostaglandin endoperoxide/thromboxane.

Arachidonic acid and U46619 cross-desensitized and the desensitization to AA, but not to U46619, was prevented by CO inhibition by MS and L8027 (Figure 4). It has been suggested (Smith & Lands, 1972; Hemler & Lands, 1980) that free radicals, formed during the oxygenation of AA, produce an irreversible inhibition or 'self deactivation' of a small portion of the CO. That desensitization of human platelets with AA in these experiments is not related to inactivation of CO or thromboxane synthetase was shown by the fact that platelets desensitized to 0.1–0.3 mM AA retained their ability to generate TxB_2 (Figure 5) and to aggregate in response to thrombin. In contrast, exposure to 1 mM AA prevented aggregation even by the high concentrations of thrombin. This suggested a toxic effect, and it was purposeless to investigate the effects of doses of AA above 0.3 mM. Further evidence that desensitization is CO-independent is provided by the homologous and heterologous desensitization evoked by U46619, which acts independently of CO or thromboxane synthetase (Di Minno *et al.*, 1979). In theory, another potential mediator of desensitization could be a lipoxygenase-derived product, such as 12-hydroperoxyeicosatetraenoic acid, which is formed by platelets when CO is blocked (Hamberg & Samuelsson, 1974; Aharony *et al.*, 1982; Croset & Lagarde, 1983). This mechanism cannot be ruled out entirely, but seems unlikely, since L8027 and MS prevented desensitization by AA under conditions where lipoxygenases are not expected to be blocked.

Desensitization by AA would appear to be mediated by one of its CO-dependent metabolite(s) which acts at a site common to U46619. The finding that the prostaglandin/thromboxane receptor antagonists TMQ and L636499 prevented desensitization by AA and U46619, indicates that either the prostaglandin endoperoxides or TxA_2 mediate desensitization by AA. Since AA-induced desensitization was not prevented by inhibition of thromboxane synthetase using dazoxiben, it appears that the formation of thromboxane is not essential for AA-induced desensitization (Figure 4; Table 2).

Platelets exposed to AA in first phase experiments secreted approximately 80% of the releasable ATP pool (determined using high concentrations of thrombin, 2.5 u ml^{-1}). Further challenge by AA or U46619 does not induce further ATP secretion, even though the residual ATP content could be readily released by thrombin. It may be argued therefore that desensitization is due to the release of granule constitutents. That AA-induced desensitization occurs independently of dense body release was demonstrated by pre-treating platelets with dazoxiben. Dazoxiben inhibits Tx synthetase, and in platelets from 'responders' also inhibits aggregation and secretion which was confirmed by electron microscopy (Figure 6). This agent, however, does not prevent desensitization by AA (Figure 6c), hence desensitization is not causally related to platelet secretion.

Platelet activation by low concentrations of thrombin was inhibited by pre-exposure to AA or to U46619, whereas responses to high concentrations of thrombin were less affected. Prevention of AA-induced desensitization with the CO inhibitors and with L636499 restored aggregation to low doses of thrombin in the second phase experiments. The CO inhibitors failed to prevent desensitization to low doses of thrombin by U46619, whereas L636499 was very effective (Table 4). These results are in accordance with the concept that CO-dependent metabolites of AA account for the responses to low, but not to high doses of thrombin (Packham *et al.*, 1973; Vargaftig, 1977; Lapetina *et al.*, 1978; Chignard *et al.*, 1979). Even though TMQ

protected against platelet desensitization by U46619 when platelets were re-exposed to AA or to U46619 itself (Table 3), this protection was not extended to the effects of the low concentration of thrombin (Table 4). We have no explanation for this discrepancy.

Platelets desensitized to AA in the presence of dazoxiben, and challenged with AA during the second phase experiments (Figure 6f) were found to be in close associations but not aggregated with inter-platelet distances greater in those platelets than in aggregated platelets (Figure 6b). Furthermore, these platelets maintained their shape and retained most of their dense bodies (compare Figure 6f with 6d and 6e). Our present hypothesis is that platelets exposed to AA in the presence of dazoxiben, which do not form TxA_2, are desensitized at the endoperoxide receptor level, thus failing to aggregate when re-exposed to AA, but are not desensitized to TxA_2 and thus retain the ability to respond to it during the second phase (Figure 6f). In this scheme, we suggest that the endoperoxides are responsible for platelet aggregation and secretion, whereas TxA_2 causes the initial cell-to-cell approximation before secretion-dependent irreversible aggregation is triggered, as reported for the early stages of primary aggregation by ADP (Charo *et al.*, 1977).

A clinical consequence of our findings is that it may be worth looking for inhibitors of platelet function acting specifically at the platelet endoperoxide receptor level, since inhibition of thromboxane synthetase alone does not inhibit platelet aggregation reliably in the majority of patients (Bertelé *et al.*, 1981; 1983). Such an approach would have the further advantage of preventing arachidonate-mediated platelet activation and the postulated endoperoxide receptor desensitization, in which case recovery from excess therapy would be facilitated.

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