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AH6809, a prostaglandin DP-receptor blocking drug on human platelets

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1 The effect of AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid) has been studied upon the anti-aggregatory and aggregatory actions of various agents on human platelets in whole blood.

2 Prostaglandin D_2 (PGD_2), BW245C, 9α,11β-PGF_2, PGI_2 and 5'-N-ethylcarboxamide adenosine (NECA) all inhibited ADP-induced platelet aggregation in whole blood. The anti-aggregatory activity of PGD_2, BW245C and 9α,11β-PGF_2 but not PGI_2 or NECA was antagonized by AH6809. NECA was antagonized by AH6809.

3 The antagonism of the anti-aggregatory activity of PGD_2 by AH6809 was concentration-related and could be overcome by increasing the concentration of PGD_2. Analysis of the data yielded an apparent pA_2 for AH6809 of 5.35.

4 At approximately 10 fold higher concentrations than those required to antagonize the action of PGD_2, AH6809 also antagonized the aggregatory effect of U-46619 in whole blood ($pA_2 = 4.45$). However, concentrations of AH6809 up to 300 μM were without effect upon either ADP- or platelet activating factor (Paf)-induced aggregation ($pA_2 < 3.5$).

5 The potency of AH6809 against PGD_2 and U-46619 was increased in a resuspended platelet preparation suggesting that the drug is extensively bound to plasma proteins. However, in resuspended platelets the specificity of AH6809 relative to that seen in whole blood was reduced since aggregation by ADP and Paf was also slightly antagonized.

6 In conclusion, AH6809 appears to be a weak but specific DP-receptor blocking drug on human platelets and should prove to be a useful drug tool for defining the involvement of endogenous PGD_2 in platelet aggregation and classifying the mode of action of anti-aggregatory prostanoids.

Introduction

Exposure of human platelets to prostaglandin (PG) E_1, I_2 or D_2 results in inhibition of platelet aggregation. These prostaglandins have been shown to inhibit aggregation by elevating platelet cyclic AMP (Mills & MacFarlane, 1974; Tateson *et al.*, 1977; Alvarez *et al.*, 1981; Feinstein *et al.*, 1981). Several studies have shown that PGE_1, PGI_2 and PGD_2 are all potent inhibitors of platelet aggregation in man (Kloeze, 1967; Smith *et al.*, 1974; Moncada *et al.*, 1976). However, on rat platelets, whereas PGE_1 and PGI_2 inhibit aggregation, PGD_2 is inactive (Whittle *et al.*, 1978). From such observations it has been suggested that PGE_1 and PGI_2 may exert their action at a common receptor site whereas PGD_2 has its action at a different receptor site, which is absent from rat platelets.

Evidence for the existence of separate receptor sites has been substantiated by the use of several different approaches. Ligand binding studies (Schafer *et al.*, 1979; Siegl *et al.*, 1979; 1980) have demonstrated specific binding sites for PGI_2 and PGD_2. In addition, agonist-specific desensitization studies (Cooper *et al.*, 1979; Miller & Gorman, 1979) have also added support for the existence of distinct receptors. In addition to these receptors mediating inhibition of platelet function, evidence also exists for the presence of receptors sensitive to thromboxane A_2 and prostaglandin H_2 on human platelets, which mediate aggregation (see MacIntyre, 1981).

A general classification for prostaglandin receptors has been proposed by Kennedy and colleagues (1982). Receptors in a range of smooth muscle preparations and human platelets have been classified according to their sensitivity to each of the naturally-occurring prostaglandins. Thus EP-, FP-

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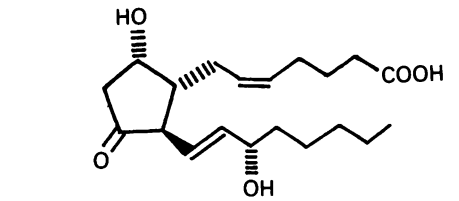

PGD_2

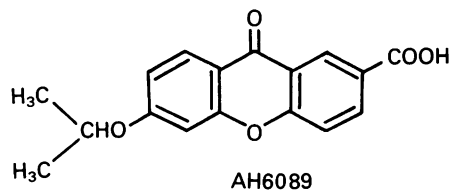

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Figure 1 The chemical structures of prostaglandin D_2 (PGD_2) and AH6809.

and TP-receptors are most sensitive to PGE_2, $PGF_{2\alpha}$ and thromboxane A_2 (TxA_2) respectively. The evidence cited above also suggests the existence of separate receptors for PGI_2 and PGD_2. These have been designated IP- and DP-receptors respectively (Kennedy *et al.*, 1982). Based upon this classification the human platelet would therefore appear to contain at least three distinct prostaglandin receptors, namely IP-, DP- and TP-receptors (MacIntyre & Armstrong, 1987).

Confirmation of this receptor classification has however been hampered by the lack of specific prostanoid receptor blocking drugs. Antagonists of the platelet DP-receptor have been reported and these have provided further evidence for the existence of separate receptor sites. For example, the phloretin derivative N-0164 (sodium-*p*-benzyl-4-[1-oxo-2-(4-chloro-benzyl)-3-phenylpropyl] phenyl phosphonate) antagonizes the anti-aggregatory effect of PGD_2 and BW245C, a PGD_2-mimetic on platelets (Town *et al.*, 1983), but not the anti-aggregatory effect of PGE_1 or PGI_2 (MacIntyre & Gordon, 1977; Whittle *et al.*, 1978; Miller & Gorman, 1979; Tynan *et al.*, 1984). AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid) (Figure 1) has been reported to antagonize the actions of prostaglandins at some receptors of the EP-type in smooth muscle preparations such as guinea-pig ileum, guinea-pig fundus and dog fundus, but not receptors of the FP- or TP-type (Coleman *et al.*, 1985). In the present study we have investigated the action of AH6809 upon the aggregatory and anti-aggregatory effects of a range of prostanoid and non-prostanoid agonists on human platelets in both whole blood and as a resuspended preparation. A preliminary account of this work has been presented to the British Pharmacological Society (Keery & Lumley, 1985).

Methods

Human blood was collected by venepuncture from the antecubital vein of healthy male volunteers who had not taken any medication for at least 10 days. The blood was anti-coagulated with trisodium citrate (1 part to 9 parts of blood) giving a final concentration in whole blood of 12.9 mM. Aliquots (0.5 ml) of blood were placed in plastic tubes which were flushed with a mixture of 5% CO_2 in air, capped and incubated in a shaking water bath (Grant Instruments, Model SS30) at 38 ± 0.5°C and allowed to equilibrate for 30 min. Aspirin was added to each tube (final concentration in whole blood 2 mM), to prevent the endogenous production of prostaglandins during the aggregatory process.

Human resuspended platelets were prepared by centrifugation of citrated whole blood (1200 *g* for 1.75 min at room temperature) containing creatine phosphate (CP: 0.5 mM) and creatine phosphokinase (CPK: 1 u ml^{-1}). The resulting platelet-rich plasma was transferred to a clean plastic container (Sterilin) and centrifuged (650 *g* for 10 min) in the presence of added prostacyclin (PGI_2: 430 nM). Following removal of the supernatant, the platelet pellet was gently resuspended in 5 ml of Krebs-Henseleit solution (composition, mM: NaCl 118, $NaHCO_3$ 25, KCl 4.75, $MgSO_4 \cdot 7H_2O$ 0.6, KH_2PO_4 1.19, D-glucose 11.1, $CaCl_2$ 1.3, pH 7.4) containing CP (0.5 mM), CPK (1 u ml^{-1}) and heparin (25 u ml^{-1}). The volume of resuspended platelet preparation was adjusted with this solution to give a final platelet count of 300–400 × 10^6 ml^{-1}. The resuspended platelets were transferred as 0.25 ml aliquots into plastic tubes containing aspirin (final concentration 20 μM), flushed with a mixture of 5% CO_2 in air before capping and stored at approximately 10°C until used. Human fibrinogen (100 μg ml^{-1}) was then added to batches (4–10) of aliquots which were incubated in a shaking water bath at 38 ± 0.5°C for 10 min before aggregation was initiated.

Platelet aggregation was quantified by counting the fall in single platelet count with an Ultra-Flo 100 whole blood platelet counter (Becton-Dickinson) according to the method of Lumley & Humphrey (1981). Briefly, the platelet count of an aliquot of whole blood or resuspended platelets was determined (control count) and aggregating agent added. The aliquot was returned to the water bath. The platelet count was then re-determined for the sample at intervals until two similar (±5%) consecutive counts were obtained or the count began to return

towards the control count. In this study aggregation was induced with either adenosine 5'-diphosphate (ADP), U-46619 or platelet activating factor (Paf). Following addition of an agonist, peak aggregation in both whole blood and resuspended platelets was usually seen at 1 min with ADP and Paf and at 5 min with U-46619. The maximum fall in platelet count was expressed as a percentage of the control count for that aliquot thus giving the percentage fall in platelet count (aggregation). By adding a different concentration of aggregating agent to different aliquots a concentration-effect curve was constructed. EC_{50} values were measured for all aggregation curves as the concentration at which a 50% fall in platelet count was obtained. Inhibitors of aggregation PGD_2, 9α,11β-PGF_2, BW245C, PGI_2, or 5'-N-ethyl carboxamide adenosine, (NECA) were preincubated with the platelets for 5 min before addition of an aggregating agent whereas AH6809, N-0164, nantradol, SC19220 or the thromboxane receptor blocking drug AH23848 (Brittain *et al.*, 1985) were preincubated for 10 min before addition of an aggregating agent. At the end of each experiment a second control curve to the aggregatory agent under study was constructed to assess any spontaneous change in platelet sensitivity over the course of the experiment. Further experimental details are described in the results section or legends to the figures.

Analysis of data

Antagonism of aggregatory agents (ADP, U-46619, Paf) was expressed as concentration-ratios (CR). These were calculated by dividing the EC_{50} in the presence of an added inhibitory agent by the mean EC_{50} obtained from the duplicate control concentration-effect curves. In the quantitative analysis of the effect of AH6809 upon the anti-aggregatory effect of PGD_2 and PGI_2, data were also expressed as PGD_2 and PGI_2 concentration-ratios. The method of calculation of these values is described in the legend to Figure 4. Agonist EC_{50}-values and all concentration-ratios are expressed as geometric mean values with 95% confidence intervals. Determination of apparent pA_2 values for AH6809 against PGD_2 and U-46619 was determined by the method of Arunlakshana & Schild (1959). Further details are given for PGD_2 in the legend to Figure 4.

Drugs

PGI_2 (1 mM) (Glaxo Group Research Limited) was dissolved in cold (4°C) 50 mM Tris buffer (pH 9.0) and subsequent dilutions made with cold (4°C) 50 mM Tris buffer (pH 8.0). PGD_2 (1 mM), 9α,11β-PGF_2 (1 mM), AH6809 (10 mM), U-46619 (10 mM; [1R-[1α,4α,5β(z),6α,(1E,3S*)]-7-[6-(3-hydroxy-1-octenyl)-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid) (Glaxo Group Research Limited) and BW245C (1 mM; 3-(3-cyclohexyl-3-hydroxypropyl)-2,5-dioxo-4-imidazolidine heptanoic acid) (Burroughs-Wellcome) were dissolved in 10% w/v sodium bicarbonate solution and 0.9% w/v sodium chloride solution (saline) added to give a final bicarbonate concentration of 1%. Subsequent dilutions were made with saline. AH23848 (1 mM; [1α(z),2β,5α]-(±)-7-[5-[[(1,1-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid) (Glaxo Group Research Ltd) and SC19220 (10 mM; 8-chloro-dibenz[b,f][1,4]oxapine-10(11H)-carboxylic acid, 2-acetyl-hydrazide) (Searle Research and Development) were dissolved in ethanol and saline was added to give a final ethanol concentration of 6%. Subsequent dilutions were made in saline. N-0164 (10 mM; Nelson Research and Development Corp.) was prepared in saline and was used as a fine suspension. Desacetyl-1-nantradol (5 mM; Pfizer) was suspended in ethanol and saline was added to give a final ethanol concentration of 9%. Adenosine 5'-diphosphate (10 mM; Sigma) was dissolved in 50 mM Tris buffer (pH 6.5) and dilutions made with saline. DL-α-Phosphatidylcholine,β-acetyl-γ-0-hexadecyl (1 mM; platelet activating factor, Paf) (Sigma) was dissolved in 0.25% w/v bovine serum albumin in saline and dilutions were made in saline. 5'-N-ethylcarboxamide adenosine (NECA; Glaxo Group Research Ltd) was dissolved in 0.1 ml of 0.5 N HCl and the concentration of drug adjusted to 1 mM with saline. Subsequent dilutions were made with saline. Creatine phosphate (100 mM), creatine phosphokinase (100 μml^{-1}) and human fibrinogen (10 $mg\,ml^{-1}$) (Sigma) were dissolved in saline. Heparin sodium BP (Mucous; Evans) was supplied as a solution containing 25,000 units ml^{-1}. Subsequent dilution of heparin was made with saline immediately before use. CS-570 (1 mM; [3aS-[2-E, 3aα, 4α(1E, 3R*, 5S*), 5β, 6aα]]-5-[hexahydro-5-hydroxy-4-(3-hydroxy-5,9-dimethyl-1,8-decardienyl)-2(1H)-pentalenylidene]-pentanoic acid, sodium salt) (Sankyo) and 3-isobutyl-1-methylxanthine (IBMX; 1 mM) (Aldrich Chemical Company Inc.) were dissolved in distilled water and dilutions prepared in saline. Aspirin (56 mM; BDH) was dissolved in 100 mM Tris buffer (pH 8.5) and dilutions prepared in saline. Value in parentheses after drug name refers to concentration of stock solutions. In the text concentrations of drugs are expressed as the concentration in whole blood or resuspended platelets. All drugs were kept on ice during an experiment with the exception of AH6809 and fibrinogen which were kept at room temperature.

Results

The effect of AH6809 upon the anti-aggregatory effect of PGD_2, BW245C, 9α,11β-PGF_2, PGI_2 and NECA in whole blood

In whole blood, ADP produced concentration-related platelet aggregation with an EC_{50} value of 0.7 (0.6–0.8) μM (mean, 95% confidence intervals, $n = 9$). A single concentration of PGD_2 (300 nM) inhibited ADP-induced aggregation; inhibition was seen as a rightward displacement of the aggregation concentration-effect curve (CR for ADP of 22.7 (4.1–127) $n = 5$) (Figure 2a). In the additional presence of AH6809 (300 μM) the inhibitory effect of PGD_2 was markedly reduced, (CR for ADP of 2.6 (1.4–5.1), $n = 5$) (Figure 2a). AH6809 produced a similar effect upon the anti-aggregatory effects of BW245C (3 nM) and 9α,11β-PGF_2 (3 μM) (Figure 2b and c). Thus in the absence of AH6809, CR values for ADP of 11.2 (4.6–27.3, $n = 5$) and 7.5 (5.2–11.0, $n = 4$) were obtained compared with 1.4 (1.0–2.0) and 1.3 (0.7–2.5) in the presence of AH6809 for BW245C and 9α,11β-PGF_2 respectively. In contrast to these effects, AH6809 (300 μM) did not antagonize the anti-aggregatory effects of PGI_2 (10 nM) (Figure 2d) or NECA (1 μM) (result not shown); in fact a small but significant ($P < 0.05$) potentiation was observed.

The reversibility of the antagonism produced by AH6809 was assessed. In the presence of AH6809 (300 μM) the anti-aggregatory effect of PGD_2 (300 nM) was virtually abolished (Figure 3). However, by progressively increasing the concentration of PGD_2 (1, 3 and 10 μM) the effect of AH6809 was overcome (Figure 3). Thus, at the highest concentration of PGD_2 used (10 μM) the antagonist effect of AH6809 had been completely overcome (compare ADP CR values of 19.5 (11.8–32.5, $n = 4$) for 10 μM PGD_2 in the presence of AH6809 with 13.9 (7.1–27.3, $n = 4$) with 300 nM PGD_2 in the absence of AH6809). Thus, in the presence of AH6809 the PGD_2 concentration had to be increased some 30 fold to produce equivalent inhibitions of ADP to that achieved in the absence of the antagonist.

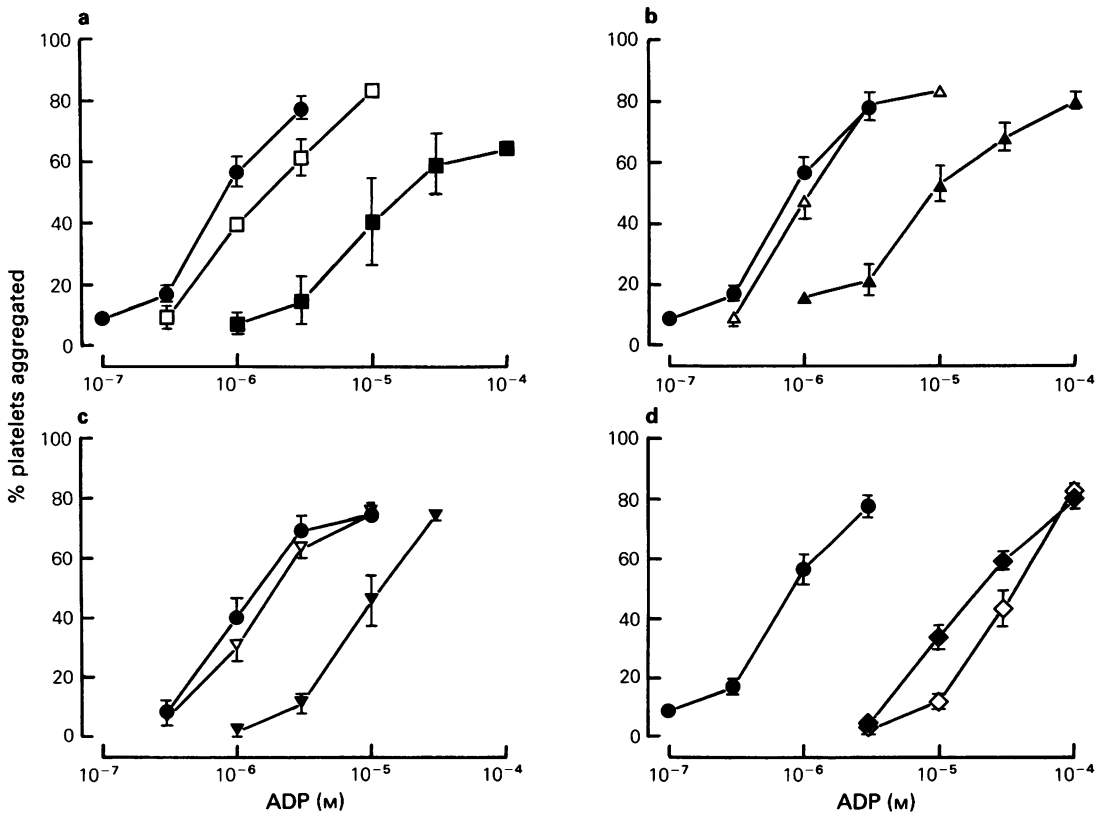

Figure 2 The effect of AH6809 (300 μM; open symbols) upon the inhibition of ADP-induced (●) human platelet aggregation in whole blood produced by (a) PGD_2 (300 nM; ■), (b) BW254C (3 nM; ▲), (c) 9α,11βPGF_2 (3 μM; ▼) and (d) PGI_2 (10 nM, ◆). Curves are the mean of 4–8 experiments with s.e.mean shown by vertical bars.

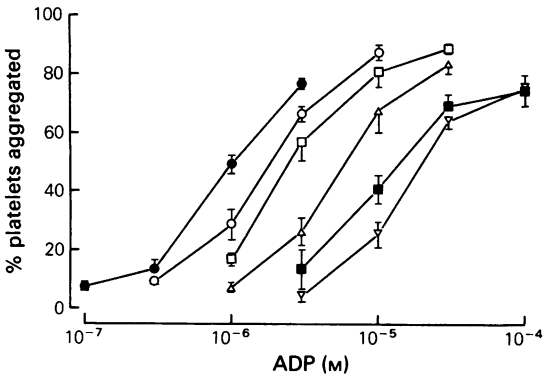

Figure 3 Determination of the reversibility of the antagonism of prostaglandin D_2 (PGD_2)-induced inhibition of human platelet aggregation by AH6809 in whole blood. ADP-induced aggregation (●) was inhibited by 300 nM PGD_2 (■). The additional presence of AH6809 (300 μM; ○) almost completely reversed the effect of PGD_2. In the continuing presence of AH6809 increasing concentrations of PGD_2 (1 μM, □; 3 μM △ and 10 μM ▽) produced a progressive rightward displacement of the ADP concentration-effect curve. Curves are the mean of 4 experiments with s.e.mean shown by vertical bars.

Analysis of the antagonism of AH6809 upon the anti-aggregatory effect of PGD_2 and PGI_2 in whole blood

Antagonism of PGD_2 by AH6809 was analysed in more detail as described in Figure 4. The PGD_2 concentration-effect curves produced by this analysis are shown in Figure 5a. At the three concentrations of AH6809 used (30, 100 and 300 μM) mean CR values for PGD_2 of 6.6 (5.1–8.4, $n = 5$), 19.9 (13.6–29.3, $n = 4$) and 35.5 (24.9–50.6, $n = 4$) respectively were obtained. Analysis of the pooled data yielded a single estimate of the apparent pA_2 value of 5.35 and a slope of the Schild regression of 0.89.

The effect of AH6809 was also assessed in the same way upon the anti-aggregatory effect of PGI_2 except that only a single concentration of AH6809 (300 μM) was investigated. As observed in the initial experiments with a single concentration of PGI_2 a slight potentiation of the anti-aggregatory effect of PGI_2 by AH6809 was obtained. This was seen as a parallel leftward displacement of the PGI_2 concentration-effect curve (Figure 5b) (CR for PGI_2 of 0.6 (0.4–0.9, $n = 4$)).

The effect of AH6809 upon ADP-, Paf- and U-46619-induced platelet aggregation in whole blood

The specificity of action of AH6809 was assessed against ADP-, Paf- and U-46619-induced aggregation. AH6809 (300 μM) was without effect upon aggregation induced by ADP or Paf (ADP EC_{50} values of 1.0 (0.7–1.2) and 1.0 (0.7–1.5) μM ($n = 11$) and individual Paf EC_{50} values before AH6809 treatment of 58 and 289 and after treatment of 60 and 288 nM respectively). Concentration-ratios for ADP and Paf are presented in Table 1.

In contrast to its lack of effect upon ADP- and Paf-, U-46619-induced platelet aggregation was antagonized by AH6809 over the concentration range 30–300 μM. In the absence of AH6809, a U-46619 EC_{50} value of 250 (120–500) nM was obtained. AH6809 produced a concentration-related rightward displacement of the U-46619 concentration-effect curve. At the highest concentration

Table 1 Comparison of the antagonistic activity of AH6809 upon the anti-aggregatory effect of PGD_2 and the aggregatory effects of U-46619, ADP and Paf in both human whole blood and a resuspended platelet preparation

	*Agonist concentration-ratio**							
	Whole blood AH6809 (μM)				*Resuspended platelets* AH6809 (μM)			
Agonist	30	n	300	n	3	n	10	n
PGD_2	6.6 (5.1–8.4)	5	35.5 (24.9–50.6)	4	6.6 (4.4–9.9)	5	24.5 (11.8–50.6)	5
U46619	1.8 (1.1–3.0)	4	9.2 (7.8–10.7)	4	3.0 (2.6–3.5)	4	9.6 (8.0–11.5)	4
Paf	0.9; 1.1‡	2	1.0; 1.0‡	2	NT†	—	1.2; 1.7; 3.6‡	3
ADP	NT†	—	1.1 (0.8–1.5)	11	1.4; 2.2; 1.4‡	3	2.2 (1.4–3.5)	4

* Agonist concentration-ratios (mean (95% confidence interval)) obtained for U46619, Paf and ADP as described in methods. Concentration-ratios for PGD_2 determined for a given concentration of AH6809 from PGD_2 concentration-effect curves as shown in Figure 4c and summarised in Figure 5a.

† N.T. = not tested.

‡ Individual agonist concentration-ratio values.

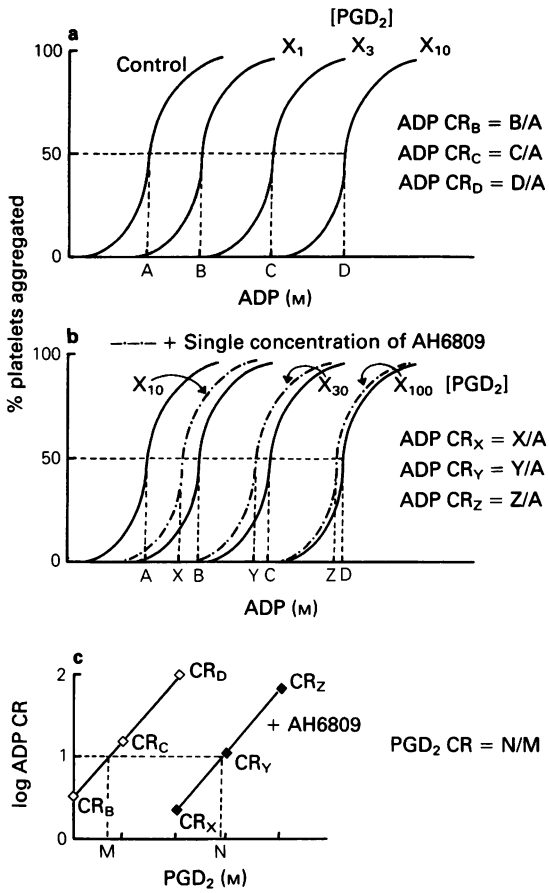

Figure 4 Construction of a prostaglandin D_2 (PGD_2) concentration-effect curve in the absence and presence of AH6809. ADP aggregation concentration-effect curves were first constructed in the absence and presence of three increasing concentrations of PGD_2 (X_1, X_3 and X_{10}). This produced a family of curves from which CR values for ADP of CR_B, CR_C and CR_D were obtained (a). Aliquots of blood were then treated with AH6809 at a single concentration. In the additional presence of this concentration of antagonist a further three ADP curves were constructed in the presence of PGD_2, the concentration of the latter being increased (e.g. X_{10}, X_{30} and X_{100}) to achieve roughly equivalent rightward displacements of the ADP curves to those observed in the absence of AH6809 (CR values of CR_X, CR_Y and CR_Z) (b). From each experiment the logarithm of the ADP CR values CR_B, CR_C and CR_D and CR_X, CR_Y and CR_Z were plotted against the corresponding PGD_2 concentration to produce a PGD_2 concentration-effect curve in the absence and presence of AH6809 (c), CR values for PGD_2 were calculated from this plot at the log ADP CR = 1.0 value. Groups of experiments (n = 4–5) were performed at each of three concentrations of AH6809 (30, 100 and 300 μM) and the PGD_2 concentration-effect curves shown in Figure 5a. CR values for PGD_2 produced by AH6809 were obtained from each experiment (c) pooled, and subjected to regression analysis by the method of Arunlakshana & Schild (1959) by plotting log PGD_2 (CR − 1) against the negative logarithm of the molar concentration AH6809 to yield a single estimate of pA_2 and slope of the Schild regression.

of AH6809 (300 μM) a mean U-46619 CR of 9.2 (7.8–10.7, n = 4) was obtained. Analysis of these data yielded a pA_2 value of 4.45 (4.27–4.63) and slope of regression of 0.97 (0.76–1.18).

The effects of AH6809 in human resuspended platelets

In studies with whole blood, a compound may be extensively bound to plasma proteins so reducing its free concentration at a receptor site. This should not be a problem in a preparation of platelets resuspended in physiological salt solution. The antagonist potency of AH6809 against PGD_2 was enhanced approximately 10 fold in resuspended platelets compared with whole blood. Thus a concentration of AH6809 of 3 μM produced an effect in resuspended platelets equivalent to that produced by 30 μM in whole blood (Table 1). Likewise, in resuspended platelets AH6809 (10 μM) produced a small potentiation of the anti-aggregatory effect of PGI_2 (CR for PGI_2 = 0.45 (0.33–0.63, n = 4)). The magnitude of this potentiation of PGI_2 was similar to that observed with 300 μM AH6809 in whole blood, again consistent with an enhanced potency of the compound in resuspended platelets.

The effect of AH6809 upon aggregation induced by U-46619, ADP and Paf was also examined in resuspended platelets. In keeping with the enhanced potency against PGD_2, the potency of AH6809 against U-46619-induced aggregation was increased some 30 fold (see Table 1). However, in contrast to its profile in whole blood, AH6809 (3 and 10 μM) was now observed to produce small rightward displacements of the aggregation concentration-effect curves to both ADP and Paf in resuspended platelets (Table 1). Clearly, non-specific actions of AH6809 presented a problem in the quantification of its action in resuspended platelets; no further studies were therefore performed in this preparation.

A comparison of the activity of AH6809 with isobutylmethyl xanthine in whole blood

In order to test whether AH6809 possessed activity consistent with inhibition of platelet cyclic AMP phosphodiesterase its effect upon aggregation was compared with that of isobutylmethyl xanthine (IBMX). U-46619 aggregation curves were constructed first alone and then in the presence of a single concentration of either the stable carbacyclin

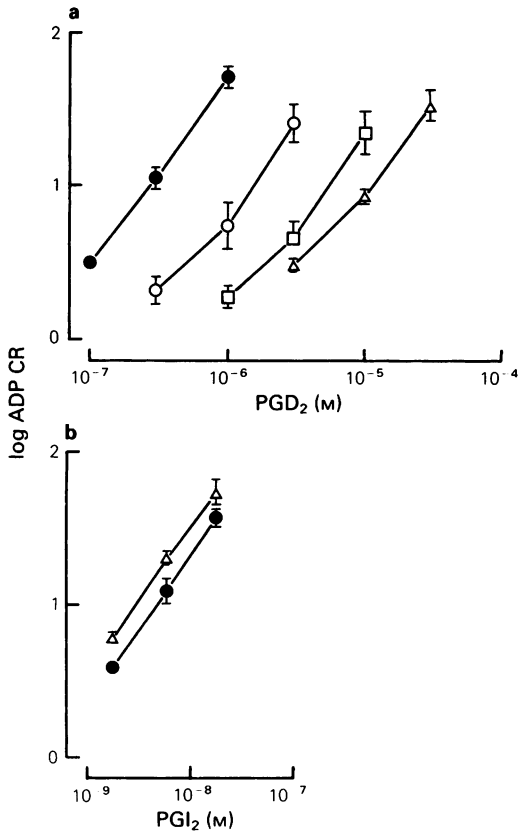

Figure 5 A summary of the effect of AH6809 upon (a) prostaglandin D_2 (PGD_2)- and (b) PGI_2-induced inhibition of ADP-induced human platelet aggregation in whole blood. Data are expressed as PGD_2 and PGI_2 concentration-effect curves which were determined as shown in Figure 4. In (a) the control PGD_2 concentration-effect curve (●) performed in the absence of AH6809 is the mean ($n = 13$), of the control curves obtained from three separate groups of experiments (as depicted in Figure 4) which each examined the effect of a different concentration of AH6809 (30 μM, ○, $n = 5$; 100 μM, □, $n = 4$; 300 μM, △, $n = 4$) upon the anti-aggregatory effect of PGD_2. In (b) only the effect of the highest concentration of AH6809 (300 μM, △) was examined upon the anti-aggregatory effect of PGI_2 (●, $n = 4$).

analogue CS-570 (30 nM) or IBMX (10 μM). In all experiments CS-570 and IBMX were incubated for 5 and 10 min respectively with the blood before addition of aggregating agent. Both CS-570 and IBMX alone produced small rightward displacements of the U-46619 aggregation curve (CR values ($n = 4$) of 1.7 (0.9–3.3) and 4.7 (0.8–26.7) respectively). However, a combination of the two drugs resulted in a profound inhibition of U-46619 aggregation (CR value of >200). Alone, AH6809 (300 μM) produced a small rightward displacement of the U-46619 curve (mean CR value of 9.4 (8.6–10.3, $n = 4$)). In contrast to the effect seen with IBMX, the additional presence of the carbacyclin analogue was without further significant effect upon U-46619 (CR value in the combined presence of AH6809 and CS-570 12.2 (9.7–15.4, $n = 4$)).

Interaction of PGD_2 with other prostanoid blocking drugs

Finally, the ability of several other prostaglandin receptor blocking drugs to antagonize the anti-aggregatory effect of PGD_2 on human platelets in whole blood was investigated and compared with the effect of AH6809. The thromboxane receptor blocking drug AH23848 (Brittain *et al.*, 1985) at a very effective but specific concentration of 1 μM was without effect upon the anti-aggregatory effect of PGD_2. AH23848 also did not modify the ability of AH6809 (300 μM) to antagonize the anti-aggregatory action of PGD_2, BW245C or 9α,11β-PGF_2. The phloretin derivative N-0164 and desacetyl-1-nantradol, which have been claimed to antagonize the anti-aggregatory effects of PGD_2 on human platelets (MacIntyre & Gordon, 1977; Horne, 1984) were also examined. Desacetyl-1-nantradol (300 μM, $n = 4$) was without effect upon the inhibition of ADP-induced aggregation produced by PGD_2 (300 nM). In contrast N-0164 (300 μM) did partially reverse the effect of PGD_2 (CR for ADP of 56.3 (9.9–321) to PGD_2 (300 nM) alone compared with 6.6 (1.7–25.4) in the presence of N-0164, $n = 4$). Neither desacetyl-1-nantradol nor N-0164 produced any significant direct effect against ADP-induced aggregation itself. In contrast to AH6809 in two experiments N-0164 produced an almost 2 fold reversal of the anti-aggregatory effect of PGI_2. Finally the EP-receptor blocking drug SC19220 (Sanner, 1969) was also evaluated ($n = 4$). At concentrations up to 300 μM, SC19220 failed to antagonize the anti-aggregatory effect of PGD_2, in fact a small potentiation (approximately 2 fold) was observed.

Discussion

The present study has demonstrated the ability of AH6809 to antagonize specifically the anti-aggregatory actions of PGD_2, but not those of PGI_2 or NECA on human platelets. In addition, the anti-aggregatory effect of BW245C, a compound which has been well characterized, both functionally and with ligand binding techniques, as acting at the human platelet DP-receptor (Town *et al.*, 1983) was antagonized by AH6809. Thus the profile of action

of AH6809 is consistent with it being a DP-receptor blocking drug. The antagonism of the anti-aggregatory effect of PGD_2 produced by AH6809 was concentration-related, but could be overcome by increasing the concentration of PGD_2. An analysis of the antagonist action of the compound in whole blood yielded an apparent pA_2 value of 5.35. However, the slope of the Schild regression was less than unity indicating that the interaction of AH6809 and PGD_2 was either not competitive and reversible or that there was interference from some complicating factor. One such factor may have been the plasma binding of AH6809. This appeared to be extensive since the potency of AH6809 was increased approximately 10 fold in a resuspended platelet preparation.

In contrast to its effects upon PGD_2 and its mimetics, AH6809 failed to antagonize the anti-aggregatory effect of PGI_2 or NECA. These agonists act at receptors (IP- and adenosine A_2-receptors respectively, Schafer *et al.*, 1979; Ukene *et al.*, 1984) which are distinct from the DP-receptor. Interestingly, rather than antagonizing the effects of these compounds a weak potentiating effect was observed with AH6809. The effect was reproducible and was greater for NECA than for PGI_2. At some 10 fold higher concentrations than those required to antagonize the anti-aggregatory action of PGD_2, AH6809 also antagonized the aggregatory effect of U-46619. However, in whole blood no such antagonist effect was observed against Paf- or ADP-induced aggregation up to concentrations of AH6809 as high as 300 μM. Thus the order of antagonist potency of AH6809 in whole blood was PGD_2 > U-46619 > ADP = Paf = 0. In contrast, in resuspended platelets, whilst the order of antagonist potency remained the same as in whole blood, a small antagonist effect was observed against ADP and Paf. The profile of action of AH6809 is clearly less complicated in a plasma containing medium such as whole blood (and most probably platelet-rich plasma) but nevertheless, the compound possesses a sufficient degree of specificity for it to be used with caution in a preparation of platelets suspended in physiological salt solution.

AH6809 has been shown to possess weak cyclic AMP-phosphodiesterase inhibitory activity in rat lung mast cells *in vitro* with an IC_{50} of 26 μM (unpublished data). Such an action might account for both the potentiation of PGI_2 and NECA and for the antagonism of U-46619-, Paf- and ADP-induced aggregation observed with the drug. In fact the rank order of susceptibility of these aggregatory agonists to an elevation of intra-platelet cyclic AMP, namely U-46619 > Paf > ADP (MacIntyre & Salzman, 1981; Armstrong *et al.*, 1983), is similar to the order observed for antagonism by AH6809. In the present study the aggregatory effect of U-46619 proved to be highly sensitive to inhibition by the combination of the cyclic AMP phosphodiesterase inhibitor IBMX and the carbacyclin derivative CS-570 (Kobayashi *et al.*, 1983). However, we were not able to demonstrate a similar potentiating effect with AH6809. Whilst not a definitive test of phosphodiesterase inhibitory activity, the lack of a potentiating effect of AH6809 argues against this mechanism of action being responsible for its action against U-46619. An alternative explanation for its effect against this agonist may be that AH6809 possesses some, albeit weak, TP-receptor blocking activity. Until this question is resolved it would be prudent to pre-block TP-receptors in experiments with AH6809 to avoid any misinterpretation of data.

Recently Liston & Roberts (1985) identified a pharmacologically active metabolite of PGD_2, 9α,11β-PGF_2, which produces contraction of vascular and airways smooth muscle and inhibition of human platelet aggregation (Roberts & Liston, 1985; Beasley *et al.*, 1986). In the present study the anti-aggregatory activity of 9α, 11β-PGF_2 has been confirmed, but is some 30 times weaker than PGD_2. The anti-aggregatory effect of 9α,11β-PGF_2 was effectively antagonized by AH6809, demonstrating the potential of AH6809 to identify anti-aggregatory agonists acting via the platelet DP-receptor. A further potential use of AH6809 is to characterize DP-receptors which may be present in other tissues. For example, Jones (1978) identified a DP-receptor mediating pressor responses in the sheep which appears distinct from that mediating inhibition of human platelet aggregation (Jones & Wilson, 1977; Jones *et al.*, 1984). In addition, Narumiya & Toda (1985) have suggested that the DP-receptor subtype mediating PGD_2-induced inhibition of human platelet aggregation is the same as that which mediates elevation of rat peritoneal mast cell cyclic AMP and relaxation of the rabbit transverse stomach strip. If this were true it would be anticipated that AH6809 would also antagonize the effects of PGD_2 and its mimetics in these biological systems.

From the data presented, AH6809 would also appear to possess a superior profile of action to previously described DP-receptor blocking drugs. The first compound to be identified was the phloretin derivative N-0164 (see Introduction). In addition to antagonizing the anti-aggregatory action of PGD_2 on human platelets (MacIntyre & Gordon, 1977) the compound exhibits thromboxane synthase inhibitory activity (Kulkarni & Eakins, 1976), antagonizes contractions produced by PGE_2 and $PGF_{2\alpha}$ on isolated gastrointestinal smooth muscle (Eakins *et al.*, 1976), and suppresses platelet aggregation to the prostaglandin endoperoxides and U-46619 (MacIntyre & Gordon, 1977). At 10 fold higher concentrations than those antagonizing PGD_2 on platelets it also

reverses the anti-aggregatory effects of PGE_1 and PGI_2 (MacIntyre & Salzman, 1981). However, in the present study, whilst N-0164 was able to reverse the anti-aggregatory effects of PGD_2, it was approximately 2 fold less potent than AH6809. It also partially reversed the anti-aggregatory effect of PGI_2. Like N-0164, AH6809 has also been shown to possess EP-receptor blocking activity (Coleman *et al.*, 1985). This EP-receptor blocking activity would not, however, be predicted to complicate the profile of action of AH6809 on human platelets since the EP-receptor blocking drug SC-19220 (Sanner, 1969) which has an EP-receptor blocking profile similar to that of AH6809 (Coleman *et al.*, 1985) was without effect upon the anti-aggregatory effect of PGD_2 in the present study. Finally a derivative of tetrahydrocannabinol, desacety-1-nantradol, has been reported to inhibit PGD_2- but not PGE_1-stimulated increases in cyclic AMP in human platelets and to compete with [^{3}H]-PGD_2 but not [^{3}H]-PGE_1 binding on human platelet membranes at concentrations as low as 10 μM (Horne, 1984). In the present study, however, we failed to show an antagonism of the anti-aggregatory effect of PGD_2 on human platelets with concentrations of desacetyl-1-nantradol up to 300 μM.

In conclusion, the current study has shown AH6809 to possess a profile of action consistent with it being a DP-receptor blocking drug. The mechanism of its potentiating effect upon NECA and PGI_2 and its antagonistic effect against U-46619, and against Paf- and ADP-induced aggregation seen only in the resuspended platelet preparation, are as yet unknown. However, by using concentrations of the drug which are specific, AH6809 can clearly distinguish compounds acting via the platelet DP-receptor from those acting at other anti-aggregatory receptors. On the current data available AH6809 would appear to be superior in its profile of action to N-0164 and represents a useful drug tool for characterization of DP-receptors. Investigation of the effect of AH6809 upon DP-receptors in other tissues is under way.

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