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pA_2 values for antagonists of platelet activating factor on aggregation of rabbit platelets

[1]Stella R. O'Donnell & Christopher J.K. Barnett

Pharmacology Section, Department of Physiology and Pharmacology, University of Queensland, St. Lucia, Brisbane, Queensland 4067, Australia

1 The relative potencies, and equilibrium dissociation constants, for nine antagonists of platelet activating factor (Paf) have been determined on rabbit platelets (in diluted platelet-rich plasma (PRP)) in experiments in which the aggregatory response to Paf was measured.

2 Log concentration-response (% maximum) curves to Paf were obtained in the absence (controls) and presence of different concentrations of each Paf antagonist drug. The antagonists shifted the Paf curves to a higher concentration range and the slopes of the Schild plots, constructed from these data, suggested that the drugs were competitive antagonists of Paf. The slopes of the Schild plots for CV-3988 and SRI 63-119 were >1.

3 The pA_2 values (pK_B values in parentheses) were: WEB 2086 7.31 (7.63); SRI 63-119 6.95; L-652,731 6.71 (6.73); BN 52021 6.38 (6.47); SRI 63-072 6.36 (6.43); CV-3988 5.87; 48740 RP 4.97 (5.07); ketotifen 4.94 (4.95); thiazinamium 4.73 (4.76).

4 This study provides, for the first time, some functional response data for Paf antagonists (pK_B values) which are in an appropriate form for use in classifying putative Paf receptors. The study also provides the comparative potencies of these Paf antagonists in inhibiting Paf-induced platelet aggregation. WEB 2086 was the most potent of the drugs examined.

Introduction

Platelet activating factor (1-0-hexadecyl/octadecyl-2-acetyl-R-glyceryl-3-phosphorylcholine, Paf, Paf-acether, AGEPC) is generated by a wide variety of cell types and has a broad range of biological activities, only one of which is platelet activation (Benveniste & Vargaftig, 1983). Some, but not all, of the biological responses to Paf appear to be mediated via Paf receptors (Godfroid & Braquet, 1986) and there is already direct evidence for specific, saturable and reversible binding of [^{3}H]-Paf to membranes from, for example, human and rabbit platelets and human lung (Hwang *et al.*, 1983; Hwang & Lam, 1986). Attempts have also been made to isolate and purify the Paf binding protein from human platelets (Valone, 1984).

Because Paf has been implicated in a wide range of pathophysiological states, a number of Paf antagonist drugs of widely differing chemical structure are already available (Braquet & Godfroid, 1986; Saunders & Handley, 1987). These drugs appear to antagonize Paf responses mediated directly via receptors and they do this in concentrations which do not antagonize responses to other stimulating agents (Braquet *et al.*, 1987). The inhibition of [^{3}H]-Paf binding to its membrane sites has already been studied for many of the Paf antagonists. From these data, it has even been speculated that there might be a heterogeneity among Paf binding sites e.g. between platelet membranes from rabbits and humans (Hwang & Lam, 1986).

Nevertheless, when the receptor systems for other autacoids, neurotransmitters or hormones have been classified pharmacologically, it has been found to be important to compare receptor binding studies with functional studies, i.e. to obtain agonist and antagonist data using measurements of the actual responses believed to be triggered by receptor activation (Kenakin, 1984). So far, with the exception of a small study by Lambrecht & Parnham (1986), there have been no Paf receptor classification studies using antagonism of Paf-induced responses. Thus, the aim of the present work was to obtain equilibrium dissociation constants for competitive antagonists of Paf in a functional study. The response selected for this

[1]Author for correspondence.

initial study was Paf-induced aggregation of rabbit platelets because there is evidence for the involvement of specific Paf binding sites in this response (Hwang *et al.*, 1983; Hwang & Lam, 1986). The pK_B values (negative log of the equilibrium dissociation constant for the Paf receptor) were determined for nine drugs, for which there was evidence in the literature that they could antagonize Paf-induced aggregation of rabbit platelets (see references in Table 2).

Preliminary accounts of this work were presented to the 2nd International Congress on Platelet-Activating Factor, Gatlinburg, U.S.A., October 1986 and to the Australasian Society of Clinical and Experimental Pharmacologists, December 1986 (O'Donnell & Barnett, 1987).

Methods

Blood was collected from the central ear artery of unanaesthetized lop-eared rabbits, into one-tenth of its volume of 3.8% sodium citrate in plastic tubes. Seven rabbits were used in the study and each provided 35–40 ml blood every 2–3 weeks. The blood was centrifuged at 300 *g* for 15 min at room temperature (about 22°C) to separate platelet-rich plasma (PRP). An aliquot of PRP was further centrifuged at 4300 *g* for 10 min at 4°C to provide platelet-poor plasma (PPP). The platelet concentration in PRP or PPP was determined using a haemocytometer and averaged $748{,}394 \times 10^6$ cells l^{-1} over 38 different PRP samples. It was zero in PPP. In each experiment the PRP sample was adjusted to the required platelet concentration of $200{,}000 \times 10^6 l^{-1}$ according to the method of Born & Foulks (1977), i.e. appropriate volumes of PPP and isotonic normal saline (NS) were used such that the final platelet suspension was always in plasma/diluted with NS in the ratio 1 : 1.5 (referred to as diluted PRP).

Aggregation responses were recorded using a Chronolog Model 330 aggregometer calibrated such that light transmittance through diluted PRP was 0% and through diluted PPP was 100%. The difference represented the maximum increase in light transmittance. Responses (increase in light transmittance) to Paf in diluted PRP took 2 to 4 min to reach a peak, depending on the concentration of Paf being used. The peak was taken as the recorded response to Paf. For each batch of diluted PRP complete concentration-response curves for Paf were obtained in the absence (control), and then in the presence, of up to 4 different concentrations of an antagonist drug. Preliminary experiments, in which data using 1 and 2 min incubation times with the antagonists were compared, showed no differences in the Schild plot data obtained from these concentration-response curves. Hence a 1 min contact time with antagonists was used.

For each curve, the log concentration of Paf was plotted against response, expressed as a % of the maximum response to Paf obtained in the control curve. This allowed changes in the maximum response due to the antagonist to be monitored. EC_{50} values, i.e. the concentration required to produce 50% of the maximum response for that curve, were interpolated. These were used to obtain values for dose-ratio (DR), i.e. ratio of the EC_{50} value in the presence of a concentration of antagonist divided by the EC_{50} value in the absence of antagonist. DR values were not corrected for changes in sensitivity of the platelets to Paf with time (see Results).

Regressions of log (DR − 1) against log [B], where [B] is the molar concentration of antagonist, were plotted, as described by Arunlakshana & Schild (1959), and are referred to as Schild plots. A linear least squares regression analysis was used to obtain the line of best fit for the combined points from a number of experiments and the slope ± s.e. mean of this regression line was obtained using methods described by Snedecor & Cochran (1967). The extrapolation of the Schild plot to log (DR − 1) = 0 gave the negative value of the pA_2 for the antagonist. Provided that the slope of the Schild plot was not significantly different from unity, the pK_B (negative log of the equilibrium dissociation constant of the receptor-antagonist complex) was calculated for each data point from the equation $pK_B = \log (DR - 1) - \log [B]$. The mean pK_B for each experiment was calculated and this was then used to obtain the mean $pK_B \pm$ s.e.mean for the total data (n = number of batches of diluted PRP). When the slope of the Schild plot is unity then the $pA_2 = pK_B$.

Drugs and chemicals

Dimethyl sulphoxide (DMSO) was obtained from Sigma (St. Louis, Mo, U.S.A.) Platelet activating factor (1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphoryl choline) was obtained either from Bachem (Bubendorf, Switzerland) or Sigma (St. Louis, Mo, U.S.A.).

The following drugs were gifts from the sources indicated: BN 52021 (3-(1,1-dimethylethyl) hexahydro-1,4,7b-trihydroxy-8-methyl-9H-1,7α (epoxymethano) - 1H, 6αH - cyclopenta [c] furo(2, 3b) [3′, 2′ : 3, 4)cyclopenta(1, 2 - d]furan - 5, 9, 12(4H) - trione)) from Dr P. Braquet, Institut Henri Beaufour, Paris, France; CV-3988 ((R,S)-2-methoxy-3-(octadecylcarbamoyloxy) propyl-2-(3-thiazolio) ethyl phosphate) from Dr M. Nishikawa, Takeda Chemical Industries, Japan; ketotifen (4-(1-methyl-4-piperidylidene) 4H-benzo [4,5]- cyclohepta [1,2-b] thiophen-10(9H)-

one hydrogen fumarate) from Dr R. Mrongovius, Sandoz, Australia; L-652,731 ((D,L)-2,5 bis (3,4,5-trimethoxyphenyl) tetrahydrofuran) from Dr J.C. Chabala, Merck, Sharpe and Dohme, New Jersey, U.S.A.; 48740 RP (3-(3-pyridyl)-1H, 3H-pyrrolo [1,2-c] thiazole-7 carboxamide) from Dr P. Sedivy, Rhone Poulenc, France; SRI 63-072 ((R,S)-3-[2[-[2 [(2 - octadecylamino - carbonyloxymethyl - tetrahydro-2-furanylmethoxy) - hydroxy-phosphinyloxy]-ethyl]-thiazolium hydroxide inner salt 4-oxide) and SRI 63-119 ((R,S)-3-[4-[(3-octadecylamino-carbonyloxy-2-methoxy) propoxy]-butyl] thiazolium bromide) from Dr D.A. Handley, Sandoz, New Jersey, U.S.A.; thiazinamium (trimethyl [1 methyl-2-phenothiazin-10-yl) ethyl] ammonium chloride) from Dr A.J. Lewis, Wyeth, Philadelphia, U.S.A.; WEB 2086 (3-[4-)2-chlorophenyl-9-methyl-6H-thieno [3,2-f][1,2,4] triazolo - [4,3-a](1,4] - diazepin-2-yl]-1-(4-morpholinyl)-1-propanon) from Dr K.H. Weber, Boehringer-Ingelheim, Ingelheim, F.R.G.

Paf was made up as a 10 mM stock solution in absolute ethyl alcohol and stored at −20°C for up to 2 months without loss of activity. Dilutions were made immediately before the experiment in NS (0.9% w/v NaCl solution) containing 0.25% bovine serum albumin. Volumes of 10 μl or, in early experiments, 50 μl were added to the cuvette such that the final concentration of Paf in 500 μl ranged between 0.1 nM and 10 μM. The final concentration of ethyl alcohol on the platelets never exceeded 0.1% and this concentration neither aggregated the platelets nor depressed Paf-induced aggregation. Antagonist drug stock solutions (10 mM) were made up either in NS (BN 52021, CV-3988, ketotifen, SRI 63-072, SRI 63-119 and thiazinamium), in DMSO (L-652,731), or in 10 mM HCl in NS (48740 RP and WEB 2086). These stock solutions were stored at −4°C for no more than 4 weeks. They were diluted on the day of the experiment either with Sorenson's phosphate buffered saline (PBS, pH 7.4) or, for L-652,731, with 50 : 50 DMSO and PBS. The antagonist was added in 10 μl and concentrations refer to the final concentration on the platelets. Dilutions of antagonist and Paf were discarded each day.

Results

Paf caused a concentration-related aggregation of rabbit platelets. An initial decrease in light transmission, a manifestation of platelet shape change, occurred for concentrations less than 10 nM. This was obscured when using either higher concentrations of Paf (because of the rapidity of the aggregation) or 50 μl volumes of a Paf solution (because of an immediate small artefactual aggregation due to dilution of the platelets). Disaggregation of the platelets, occurring after the peak, was seen for low, but not high, concentrations of Paf.

The mean $-\log EC_{50}$ value for Paf was 8.91 ± 0.04 and the mean maximum increase in light transmittance was $70.38 \pm 2.07\%$ ($n = 32$). Log concentration-response curves to Paf, obtained at 45 min intervals during the time required to carry out an experiment with 4 concentrations of an antagonist, were superimposed. For example, in 5 experiments, the mean $-\log EC_{50}$ for Paf was 8.78 ± 0.08 for the first curves and 8.70 ± 0.10 for curves done approximately 4 h later (not significantly different, paired t test). There was no change in maximum light transmittance during this time. Thus, the sensitivity of the platelets to Paf did not change with time and no correction factors for this were necessary.

None of the antagonists caused any aggregatory response when added to the platelet suspension. Control Paf curves were carried out in the presence of the appropriate solvent/diluent for each antagonist. This was particularly important when DMSO was used (i.e. for L-652,731 and for some preliminary experiments with BN 52021) because DMSO reduced the sensitivity of the platelets to Paf ($-\log EC_{50}$ in the absence of DMSO was 8.93 ± 0.11 and in the presence of DMSO was 8.38 ± 0.13 ($n = 6$), significant reduction by DMSO, $P < 0.001$, paired t test). The maximum response to Paf was also reduced to $85.7 \pm 2.4\%$ ($n = 6$) of that in the curve without DMSO. This agrees with recent results obtained with DMSO on human platelets (Lehur & Curtis-Prior, 1987).

All the antagonists caused a parallel displacement of the Paf log concentration-response curve to a higher concentration range. There was no depression of the maximum response to Paf except with the highest concentration of L-652,731 (10 μM) and of 48740 RP (700 μM). Analysis of these curves gave the Schild plot data summarized in Figure 1. For CV-3988 and SRI 63-119, the slopes of the Schild plots were significantly greater than unity. For all the remaining antagonists the slopes were not significantly different from 1 (Table 1), i.e. the data satisfied this condition for competitive antagonism. The pA_2 values determined experimentally from the Schild plots and the mean of the calculated pK_B values, for those antagonists where evidence of competitive antagonism was obtained, are summarized in Table 1.

Discussion

The present functional response study has provided presumptive evidence that the drugs WEB 2086,

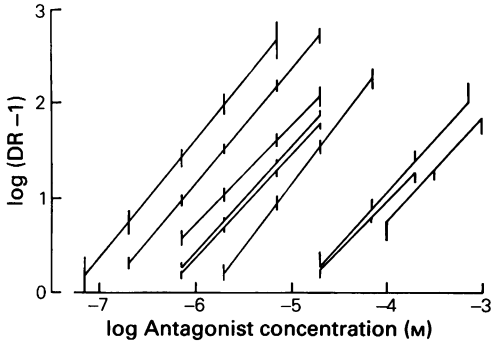

Figure 1 Schild plots for antagonist drugs against Paf-induced aggregation of rabbit platelets. The plots are (from left to right) for WEB 2086, SRI 63–119, L-652,731, BN 52021, SRI 63-072, CV-3988, 48740 RP, ketotifen and thiazinamium. The plots represent calculated regression lines of best fit through the combined data points from the experiments shown in Table 1. The vertical lines represent the s.e. mean around the estimated values of log (DR − 1) at those points corresponding to the antagonist concentrations used for that particular antagonist. Extrapolation of these calculated lines to log (DR − 1) = 0 provided the pA_2 values shown in Table 1. The slopes of these lines are also shown in Table 1.

L-652,731, BN 52021, 48740 RP, ketotifen and thiazinamium are competitive antagonists of Paf-induced aggregation of rabbit platelets, in that the slopes of the Schild plots were not significantly different from 1. The slopes of the Schild plots for CV-3988 and SRI 63-119, which are both analogues of Paf, were greater than 1. This was not due to a lack of equilibration of these antagonists with the receptors since the slope was still greater than 1 if the contact time with the antagonist was increased. It is not known whether these drugs cause, for example, a non-specific depression of platelet aggregation at high concentrations, stimulate an alternative aggregatory mechanism at low concentrations or whether there might be a removal process for them which becomes saturated at high concentrations of the drug (Kenakin & Beek, 1987).

Equilibrium dissociation constants (for the drug-receptor complex), and/or pA_2 values, for the above Paf antagonists were obtained. pA_2 values for kadsurenone have previously been obtained (Shen *et al.*, 1985; Lambrecht & Parnham, 1986) but, for all other Paf antagonists, only IC_{50} values (concentrations of antagonists to produce 50% inhibition of the response to a standard concentration of Paf) are available in the literature. These values do provide an order of potency for the drugs (Table 2) which parallels that found in the present study (Table 1). However, in relation to competitive enzyme inhibition, Cheng & Prussof (1973) have demonstrated that the IC_{50} value does not equal the K_i value (dissociation constant for the enzyme-inhibitor complex) since the IC_{50} value depends on the concentration of the substrate and the Michaelis constant. This will also be the case for competitive drug receptor antagonists, but for the relationship between IC_{50} and K_B values. Also, their recommendation that IC_{50} values should not be compared between laboratories, unless identical assay conditions are used, will also apply to receptor studies. Thus, the IC_{50} values in Table 2 cannot validly be compared whereas pK_B values from the present study can be compared, not only with each other, but also with values from other laboratories.

On the other hand, K_B values are only valid if determined under the correct experimental condi-

Table 1 Antagonism of Paf-induced aggregation of rabbit platelets in platelet-rich plasma (PRP): normal saline (1 : 1.5)

Antagonist	*Schild plot slope* (± s.e. mean) (n_1)	pA_2	pK_B (± s.e. mean) (n_2)
WEB 2086	1.23 ± 0.16 (16)	7.31	7.63 ± 0.08 (5)
SRI 63-119	1.22* ± 0.06 (10)	6.95	# (3)
L-652,731	1.03 ± 0.10 (8)	6.71	6.73 ± 0.05 (4)
BN 52021	1.10 ± 0.04 (12)	6.38	6.47 ± 0.04 (3)
SRI 63-072	1.06 ± 0.07 (7)	6.36	6.43 ± 0.06 (3)
CV-3988	1.31* ± 0.11 (10)	5.87	# (5)
48740 RP	1.08 ± 0.18 (10)	4.97	5.07 ± 0.14 (4)
Ketotifen	1.01 ± 0.20 (8)	4.94	4.95 ± 0.10 (3)
Thiazinamium	1.04 ± 0.26 (7)	4.73	4.76 ± 0.16 (3)

Slopes of Schild plots, pA_2 values and mean pK_B values are shown.
n_1 = number of data points; n_2 = number of different PRP samples.
The pK_B values were not calculated because the slope of the Schild plot was significantly greater than 1.
* Slope significantly greater than 1 ($P < 0.05$).

Table 2 Some IC_{50} values from the literature for antagonist drugs in inhibiting Paf-induced aggregation of rabbit platelets

Antagonist	*IC_{50}* (μM)	*Paf concentration* (nM)	*Reference*
SRI 63-119	0.4	4.8	Winslow *et al.* (1987)
BN 52021	0.58	2.5	Braquet (1985)
	0.14–0.26	0.25–0.5	Baroggi *et al.* (1988)[1]
SRI 63-072	4.7	4.8	Winslow *et al.* (1987)
CV-3988	7.8	30	Terashita *et al.* (1983)
	4.8	4.8	Winslow *et al.* (1987)
48740 RP	40	18	Sedivy *et al.* (1985)
	16	9 to 18	Floch *et al.* (1985)
Ketotifen	175	14 to 28	Criscuoli *et al.* (1986)
Thiazinamium	225	14 to 28	Criscuoli *et al.* (1986)

Platelets were in platelet-rich plasma (PRP) unless indicated.
[1]Platelets were washed and resuspended.

tions, and these have been well delineated for Schild plot experiments using responses of isolated tissues (Furchgott, 1972; Kenakin, 1984; O'Donnell, 1985). Since the slopes of the Schild plots in the present study were not less than unity, it can be deduced that a saturable uptake process for Paf in platelets is unlikely to be complicating the data (Kenakin, 1984). The influence, if any, of proteins and enzymes present in the diluted plasma is currently being addressed by carrying out experiments in rabbit washed platelets. However, a recent study (Stewart & Dusting, 1988) indicates that the pA_2 values, for WEB 2086 and BN 52021 at least, might not be different from those in PRP.

In summary, this study has provided, for the first time in a functional response study, data for a series of competitive Paf antagonists which can be compared with similar data from other laboratories using other Paf-receptor-induced responses. Such constants are of importance if postulated Paf receptor subtypes (Hwang & Lam, 1986; Lambrecht & Parnham, 1986) are to be classified and the possible selectivity of antagonists determined.

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