Antagonism of platelet activating factor-induced chemiluminescence in guinea-pig peritoneal macrophages in differing states of activation

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1 The effects of the platelet activating factor (Paf) antagonists alprazolam, BN 52021, kadsurenone, L 652,731 and SRI 63119 have been studied on Paf-induced chemiluminescence (CL) of guinea-pig, *C. parvum*-activated peritoneal macrophages *in vitro*.

2 All antagonists produced a shift to the right in the dose-response curve to Paf ($0.001\text{--}10\ \mu\text{mol l}^{-1}$). Schild plots for BN 52021, L 652,731, kadsurenone and SRI 63119 were linear, but only for BN 52021 and kadsurenone did the mean slope not differ significantly from unity. Mean pA_2 values for BN 52021 and kadsurenone were 6.60 ± 0.05 and 6.41 ± 0.14 (mean $\pm$ s.e.mean) respectively. Calculation of IC_{50} values for all antagonists (at $0.1\ \mu\text{mol l}^{-1}$ Paf) gave an order of potency: L 652731 > kadsurenone $\geq$ BN 52021 > alprazolam > SRI 63119.

3 When individual pA_2 values for BN 52021 and kadsurenone were plotted against the maximal CL response to Paf of cell suspensions in the absence of antagonist (reflecting the degree of activation of the macrophages by the *C. parvum*), it was found that the affinity of both antagonists for macrophage Paf receptors remained relatively constant irrespective of the activation state of the cells.

4 We conclude that activation of guinea-pig peritoneal macrophages does not account for the increased affinity for macrophage Paf receptors previously observed for kadsurenone. Kadsurenone and BN 52021 presumably bind to a site on Paf receptors which is not affected by the activation process, while alprazolam and SRI 63119 are non-specific antagonists. The reason for the difference between the competitive nature of kadsurenone and its structural analogue L 652,731 is unclear.

Introduction

Platelet activating factor (Paf) is formed on stimulation of a variety of inflammatory and immunocompetent cells and stimulates neutrophil, eosinophil, lymphocyte and macrophage functions (Braquet *et al.*, 1987). Among the responses of macrophages to stimulation by Paf are increased glucose consumption and the generation of reactive oxygen species (the 'oxidative burst'), increased interleukin 1 formation and release of arachidonic acid metabolites (Hartung *et al.*, 1983; Hartung, 1983; Hayashi *et al.*, 1985; Barrett *et al.*, 1987; Huang *et al.*, 1988; Stewart & Dusting, 1988). The ability of Paf to stimulate the macrophage oxidative burst is closely related to the presence of specific binding sites on the cell membrane, since rat and mouse peritoneal macrophages barely respond to Paf and cells from these species do not exhibit specific Paf-binding (Parnham & Leyck, 1983; Kudo *et al.*, 1986;

Parnham, 1988). While resident, non-activated peritoneal macrophages from guinea-pigs also weakly respond with an oxidative burst to Paf and do not exhibit specific [^3H]-Paf binding sites, immunologically activated or elicited macrophages from this species are particularly sensitive with regard to both induction of the oxidative burst and specific binding (Hartung *et al.*, 1983; Kudo *et al.*, 1986).

Recently, we have shown that the affinity (pA_2) of the Paf receptor antagonist, kadsurenone, for receptors on guinea-pig *Corynebacterium parvum*-activated, peritoneal macrophages (determined by measuring the oxidative burst to Paf in terms of chemiluminescence) is approximately 100 fold greater than that for Paf receptors on pig peripheral blood leucocytes (Lambrecht & Parnham, 1986). This led us to propose the existence of a high affinity Paf receptor subtype, Paf_2 , on guinea-pig activated

macrophages, a proposal supported by recent studies by other authors (Stewart & Dusing, 1988). Subsequent to our original finding we have studied the affinities of a variety of Paf receptor antagonists for Paf₂ receptors on guinea-pig activated macrophages, using kadsurenone as a reference. During these experiments it became apparent that the previously published high pA₂ value for the action of kadsurenone on this cell type in many cases could not be achieved. Postulating that the high affinity of kadsurenone previously observed (Lambrecht & Parnham, 1986) was related to the high degree of activation of the macrophages by the heat-killed *C. parvum*, we compared the calculated affinities of the various Paf antagonists for macrophage Paf receptors with the activation state (determined as the maximal chemiluminescence responses of individual cell suspensions) of the cells. A preliminary account of some of these data has been published (Bittner *et al.*, 1988).

Methods

Male Pirbright white guinea-pigs (200–300 g, Süd-deutsche Versuchstierfarm, W. Germany), kept under constant light and temperature with food and water *ad libitum*, each received an i.p. injection of 1.5 ml heat-killed *Corynebacterium parvum* (7 mg ml⁻¹, Deutsche Wellcome, Burgwedel) and were killed with CO₂ 4, 8 or 12 days later. Three different time points were chosen for harvest of the cells to obtain a broad distribution of macrophage activation states in order to facilitate the determination of correlations with antagonist affinities. Harvesting of cells by peritoneal lavage, resuspension in buffer and stimulation with synthetic hexadecyl-*rac*-Paf (Nattermann, Cologne) in the presence of the chemiluminescence (CL)-enhancer, luminol, in a six-channel Berthold Biolumat LB 9505 were carried out as described previously (Lambrecht & Parnham, 1986). Dose-response curves to Paf in the presence and absence of increasing concentrations of individual receptor antagonists (alprazolam, 1–10 µmol l⁻¹, BN 52021, 0.32–10 µmol l⁻¹; L 652,731 0.1–10 µmol l⁻¹; kadsurenone, 1–10 µmol l⁻¹; SRI 63119, 0.32–10 µmol l⁻¹) were performed on the same cell suspension, each cell suspension being prepared from a single guinea-pig. Each antagonist concentration was tested against the complete Paf dose-response curve once for each cell suspension. Because of the limitation of the number of cells, only one antagonist was tested per cell suspension. Dose-ratios (DR) for antagonist-induced shifts in the dose-response curves to Paf were estimated, Schild plots constructed and pA₂ values calculated as described previously (Lambrecht & Parnham, 1986). In addition, because the Schild plots were not all linear, antagonist potencies were

also compared by calculating for each antagonist concentration percentage inhibition of CL responses to 0.1 µmol l⁻¹ Paf. This concentration was chosen as it was a sub-optimal concentration lying on the linear part of the dose-response curve to Paf. IC₅₀ values were determined for each antagonist from regression lines fitted for log concentration of antagonist against percentage inhibition.

In a further series of experiments the maximal activation state (MAS) of cell suspensions from individual animals was determined as the absolute CL (c.p.m.) generated at the peak of the Paf dose-response curve in the absence of antagonist. The MAS for the cell suspension was plotted against the pA₂ for the antagonist determined on that cell suspension (at one antagonist concentration per suspension) and the regression analysis for all such plots obtained for each antagonist carried out using an IBM personal computer programme (EPISTAT).

Drugs

Antagonists used were kadsurenone and L 652,731 ((±)-2,5-bis(3,4,5-trimethoxyphenoxy)tetrahydrofuran-) (kindly provided by Dr T.Y. Shen, Merck, Sharpe & Dohme, Rahway, U.S.A.), BN 52021 (ginkgolide B) (kindly provided by Dr P. Braquet, IPSEN-Beaufort, Paris, France), SRI 63119 (R,S)-3-[4-[(3-octadecylaminocarbonyloxy-2-methoxy)-propoxy]butyl]thiazolium bromide (kindly provided by Dr C. Winslow, Sandoz, Hanover, U.S.A.) and alprazolam (Upjohn, W. Germany).

Results

Antagonism of Paf-induced chemiluminescence

All five Paf receptor antagonists tested produced a shift to the right in the dose-response curve to Paf on guinea-pig activated macrophages (Figures 1 and 2). At the lowest concentration, BN 52021 appeared to reduce the maximal response to Paf, while alprazolam failed to produce a parallel shift in the dose-response curve, rather exhibiting greater antagonism at lower Paf concentrations (Figure 2). The remaining antagonists all produced parallel shifts in the Paf dose-response curve.

Only kadsurenone and BN 52021 yielded Schild plots which did not differ significantly from unity (Figure 3; Table 1). For the remaining three antagonists, the lack of a slope of unity and for alprazolam, the lack of linearity of the Schild plot (data not shown), meant that the conditions for competitive antagonism were not fulfilled and pA₂ values could not be used. Comparative antagonist potency was therefore determined on the basis of IC₅₀ values for

Figure 1 Inhibition by kadsurenone (a) and L 652,731 (b) of the chemiluminescence (CL) responses of guinea-pig, *C. parvum*-activated peritoneal macrophages to Paf. Complete dose-response curves were obtained on individual cell suspensions from single guinea-pigs in the absence (●) and in the presence of antagonists at concentrations of 1.0 (■) and 10.0 $\mu\text{mol l}^{-1}$ (▲). Each point is the mean of 3–6 observations obtained from 6 animals; vertical lines show s.e.mean. For reasons of clarity, all antagonist concentrations used are not given.

inhibition of CL responses to 0.1 $\mu\text{mol l}^{-1}$ Paf. As shown in Table 1, the order of potency was: L 652,731 > kadsurenone $\geq$ BN 52021 > alprazolam > SRI 63119, kadsurenone and BN 52021 exhibiting the clearest concentration-response relationship, as indicated by the regression coefficients. Since the Schild plots for kadsurenone and BN 52021 have properties consistent with competitive antagonism of Paf, their calculated pA_2 values (Table 2) apparently reflect their affinities for Paf receptors on guinea-pig activated macrophages and confirm the IC_{50} data showing identical affinities.

Relationships between antagonist affinity and macrophage activation state

The similarity in the apparent affinities of BN 52021 and kadsurenone for macrophage Paf receptors was

Figure 2 Inhibition by SRI 63119 (a), alprazolam (b) and BN 52021 (c) of the CL responses of guinea-pig, *C. parvum*-activated peritoneal macrophages to Paf. Complete dose-response curves were obtained on individual cell suspensions from single guinea-pigs in the absence (●) and in the presence of antagonists at concentrations of 1.0 (■) and 10.0 $\mu\text{mol l}^{-1}$ (▲) for BN 52021 and SRI 63119 and 3.0 (■) and 30.0 $\mu\text{mol l}^{-1}$ (▲) for alprazolam. Each point is the mean of 2–6 observations from 6 animals; vertical bars show s.e.mean. For reasons of clarity, all antagonist concentrations used are not given.

Figure 3 Least squares fit Schild plots for antagonism by kadsurenone (a) and BN 52021 (b) of Paf (10^{-10} – 10^{-4} mol l $^{-1}$)-induced CL responses of guinea-pig activated peritoneal macrophages. Each point is the mean value obtained from 3–6 separate observations each made on a different cell suspension, each cell suspension harvested from a different animal; s.e.mean shown by vertical bars. Ordinate scale: log (DR – 1) where DR = dose-ratio measured from the displacement of the Paf dose-response curve by the respective antagonist. Abscissa scale: negative logarithm of the molar concentration of the respective antagonist.

supported when their affinities (pA_2 values) were correlated with the maximal activation state (MAS) of individual cell populations (Figure 4). Both compounds exhibited a similar affinity, irrespective of the MAS of the macrophages (Table 2).

Discussion

The data presented in this paper indicate that responses of guinea-pig activated macrophages to Paf can be antagonized by a variety of structurally different antagonists, and that the affinities of at least two compounds which exert competitive antagonism of the macrophage Paf receptors do not vary according to the activation state of the macrophages.

A number of reports have appeared in the literature recently suggesting that subtypes of Paf receptors can be detected on different cell membranes. For instance, Hwang & Lam (1986), using [3H]-Paf-binding, reported that the order of potency of seven different Paf receptor antagonists differs between human and rabbit platelets, while Hwang (1988) found different orders of potency when measuring antagonism of Paf binding and signal transduction parameters in human leukocytes and platelets. Hwang (1988) suggested that the differences in affinity which we observed (Lambrecht & Parnham, 1986) for kadsurenone between guinea-pig activated peritoneal macrophages and pig peripheral blood leukocytes stimulated with Paf may have been due to such species and/or intercellular differences in Paf

Table 1 Characteristics of the antagonism by various Paf receptor antagonists of the chemiluminescence response to Paf in guinea-pig activated peritoneal macrophages

	Inhibitory-potency			Schild plot regression slopes
	IC_{50} (μ mol l $^{-1}$)	Relative potency	Regression coeff. (r)	
Alprazolam	161.00 (21)	383.3	0.505†	-0.365 ± 0.115 (10)**
BN 52021	6.08 (23)	14.3	0.662††	-0.925 ± 0.117 (24)
L 652731	0.42 (24)	1.0	0.426†	-0.625 ± 0.024 (24)*
Kadsurenone	5.25 (16)	12.3	0.773††	-0.960 ± 0.064 (15)
SRI 63119	311.00 (17)	731.0	0.535†	-0.574 ± 0.056 (13)*

Values are given as means $\pm$ s.e.mean; numbers in parentheses indicate the number of experiments.

Significance of difference of correlation from zero: † $P < 0.05$, †† $P < 0.001$. Significance of difference from unity:

* $P < 0.05$, ** $P < 0.01$.

The no. of observations for IC_{50} determinations differ from those for Schild plots, because in some experiments only one or two antagonist concentrations were used. For kadsurenone, in 3 experiments, the dose-ratio at 1μ mol l $^{-1}$ antagonist was too small to permit accurate calculation for the Schild plot.

Table 2 Relationship between antagonist affinity and macrophage activation state (MAS) for kadsurenone and BN 52021

	Mean pA_2	n	Correlation coefficient (r) for MAS vs pA_2
Kadsurenone	6.41 ± 0.14	14	0.461
BN 52021	6.60 ± 0.05	13	-0.020

Values are given as means $\pm$ s.e.mean.

Significance of difference of correlation from zero:

$P > 0.05$ in each case.

receptor characteristics. In any case, our present data demonstrate that the affinity of kadsurenone, like that of BN 52021, for Paf receptors on the total population of guinea-pig peritoneal macrophages appears to be independent of the state of cellular activation.

Activation of mouse peritoneal macrophages by *C. parvum* has been shown to enhance markedly the expression of surface Fc (IgG_{2b}) receptors and Ia antigens and to decrease slightly the expression of surface C3 receptors for complement (Glass *et al.*, 1984). Recently, Walker & Beelen (1988) have reported that in rat peritoneal macrophages, fractionation reveals discrete groups of cells with markedly increased expression of Fc receptors and phagocytic capacity. At present, we cannot rule out the possibility that in discrete groups of cells in our study the affinity of kadsurenone for Paf receptors may have been increased, but this was not detectable in the total peritoneal cell population. Different proportions of such discrete groups of cells may have accounted for the high affinity for kadsurenone observed in our previous study (Lambrecht & Parnham, 1986) as well as for our failure to confirm preliminary findings on the relation between cell activation and Paf receptor antagonist affinity (Bittner *et al.*, 1988). The fact that BN 52021 exhibited similar mean pA_2 values to kadsurenone on Paf-stimulated guinea-pig macrophages and did not show a change in affinity with increasing cell activation suggests that BN 52021 binds to a similar site on the Paf receptor to kadsurenone.

Recently, Stewart & Dusting (1988) have reported that BN 52021, L 652,731 and another Paf receptor antagonist WEB 2086 all exhibit lower affinities (pA_2 values) for guinea-pig resident macrophage receptors than for platelet and leukocyte Paf receptors. These authors used Paf-stimulated macrophage prostacyclin production as their parameter. Guinea-pig resident macrophages barely respond to Paf with an oxidative burst, in contrast to *C. parvum*-activated cells (Hartung *et al.*, 1983), while specific ³H-Paf

Figure 4 Correlations for (a) kadsurenone and (b) BN 52021 between pA_2 values determined on different guinea-pig activated macrophage suspensions and the maximal activation state (MAS; measured as the peak CL response to Paf in the absence of antagonist) of the individual cell suspension from which the pA_2 value was calculated. Each point is the mean of 1–3 determinations of pA_2 values calculated for different antagonist concentrations on the same cell suspension.

binding sites could not be detected on resident cells (Kudo *et al.*, 1986). The possibility thus arises, as suggested by Stewart & Dusting (1988), that cell membrane-associated Paf may exert biological effects, independently of receptor binding, for instance on resident macrophages. Certainly, Paf exhibits a high degree of non-specific binding to a number of cells but no report has appeared in which this non-specific binding has been correlated with a biological effect.

In a study on permanent macrophage cell lines, Valone (1988) reported that HEL, U-937 and HL-60 cells bound 'substantial quantities' of Paf, but he discussed this binding as being non-receptor-mediated

on the basis of antagonist effects. Interestingly, he observed that kadsurenone did inhibit [^3H]-Paf binding to these cells, but BN 52021 did not. Also in P388D₁ mouse macrophage-derived cells BN 52021 was 14 fold less potent as an inhibitor of Paf binding in comparison to kadsurenone (Valone, 1988). Such a distinction does not appear to hold for guinea-pig peritoneal macrophages.

The fact that in our experiments the Schild plot for kadsurenone was linear with a slope which was not significantly different from unity, while its analogue L 652,731 produced a plot with a slope different from unity requires explanation. It seems likely that L 652,731, while more potent than kadsurenone, may require a longer period of time to achieve thermodynamic equilibrium with the Paf receptors as has been proposed for other receptor types (Kenakin, 1987). Alternatively, the specificity of action of the two antagonists may differ. Stewart & Dusting (1988) were also unable to calculate a pA_2 value for L 652,731 with guinea-pig resident macrophages, though, in their case, this was because of the low potency of the compound on these cells. The apparent non-competitive nature of the antagonism by alprazolam as reflected by the non-linear Schild plot for this compound, may be due to more than one mechanism of antagonism of the Paf-induced CL response as has been proposed for other receptor types (Kenakin, 1987). Alprazolam is a triazolobenzodiazepine (Kornecki *et al.*, 1984) and is used clinically as an anxiolytic and hypnotic agent. Recently, mouse peritoneal macrophages have been shown to exhibit saturable binding sites for the benzodiazepine, flunitrazepam (Zavala *et al.*, 1985), while a benzodiazepine (diazepam) has been reported to inhibit immune responses in mice *in vivo* (Descotes *et al.*, 1982). Consequently, the antagonism by alprazolam of Paf-induced CL responses of guinea-pig peritoneal macrophages may have been due at least to interaction of the antagonist with both Paf and benzodiazepine receptors. Similarly, SRI 63119, a compound structurally related to Paf (Handley *et al.*, 1986), probably exerted a non-specific action on macrophage CL. In fact, in another recent study, SRI 63119 also failed to produce a Schild plot with a slope of unity, when tested on rabbit platelet aggregation (O'Donnell & Barnett, 1988).

Lack of linearity and of slopes of unity of Schild plots complicate direct comparison of antagonist potencies between different studies. O'Donnell & Barnett (1988) have drawn attention to the fact that only with pA_2 values can direct comparisons of antagonist potencies be made, IC_{50} values obtained under different conditions only permitting orders of

potency to be assessed. In this respect, the pA_2 value we observed for BN 52021 (6.6) corresponds closely to the value of 6.85 obtained by Stewart & Dusting (1988) on rabbit washed platelets and of 6.38 obtained by O'Donnell & Barnett (1988) on rabbit platelet-rich plasma. The binding sites for BN 52021 and the Paf receptors of these cells, therefore would appear to be similar. The mean pA_2 of 6.41 we observed in the present study for kadsurenone correlates closely with the values obtained by Shen *et al.* (1985) of 6.28 for rabbit platelet aggregation and 6.32 for human leukocyte aggregation and the value of 6.16 obtained by Lambrecht & Parnham (1986) for pig leukocyte aggregation.

Comparison of orders of potency of four of the antagonists we studied reveals that in most cell systems L 652,731 is more potent than kadsurenone (Table 1; Hwang & Lam, 1986), which is as potent or more potent than BN 52021 (Hwang & Lam, 1986; O'Donnell & Barnett, 1988; Stewart & Dusting, 1988; Ward & Westwick, 1988). The exception is guinea-pig resident macrophages in which BN 52021 appeared to be more potent than L 652,731, but did not fulfil the criteria for a competitive inhibitor (Stewart & Dusting, 1988). Although we observed that SRI 63119 was a weak antagonist on activated macrophages, O'Donnell & Barnett (1988) found it to be a potent Paf antagonist on rabbit platelets. However, the pA_2 value given is dubious since the compound did not give a Schild plot with a slope of unity. In addition, the authors used platelet-rich plasma in which endogenous Paf may have interfered with the activity of the structurally-related antagonist.

In conclusion, we have shown that structurally different Paf receptor antagonists can be differentiated on the basis of their effects on Paf receptors on guinea-pig activated macrophages. The finding that kadsurenone and BN 52021 do not exhibit increasing affinity for Paf receptors, with increasing macrophage activation, would appear to rule out cell activation as an explanation for our previously reported finding on the differential affinity of kadsurenone for Paf receptors on macrophages and neutrophils. Cell fractionation studies are required to determine whether discrete fractions of peritoneal macrophages differ with regard to Paf antagonist affinity.

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