Albumin inhibits platelet-activating factor (PAF)-induced responses in platelets and macrophages: implications for the biologically active form of PAF

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1 Platelet-activating factor (PAF) binds with high affinity to albumin leading Clay *et al.* (1990) to suggest that the active form of PAF is the albumin-PAF complex.

2 In the present study the proposal that albumin-bound, rather than monomeric PAF, is the active form of PAF at PAF receptors was critically evaluated by examining the effect of albumin on the potency of PAF in isolated platelets and macrophages.

3 Bovine serum albumin inhibited concentration-dependently PAF-induced responses in platelets and macrophages. The most probable explanation of this finding is that BSA reduced the concentration of free PAF.

4 Thus, we conclude that free PAF, rather than the albumin-PAF complex is the active form. Consequently, local concentrations of albumin will influence profoundly the potency of endogenously released PAF. Moreover, estimates of the affinity of PAF for PAF receptors made in buffers containing BSA, underestimate the true affinity of PAF for its receptors by approximately 3 orders of magnitude.

Keywords: Platelet-activating factor; albumin; platelet; macrophage; pharmacodynamics; receptors.

Introduction

Platelet-activating factor (PAF) is a phospholipid-derived mediator of allergy and inflammation (Braquet *et al.*, 1987). It is now established that PAF acts by stimulating stereospecific, high affinity receptors on a wide range of cell types including smooth muscle, leukocytes, endothelium and neurones (Hwang, 1988).

Albumin acts as a carrier of a wide range of substances in the blood, including a number of fatty acids (Spector, 1975). Albumin has a number of binding sites for fatty acids and exerts complex effects on the synthesis and metabolism of prostaglandins and PAF (Heinsohn *et al.*, 1987). PAF is known to be influenced by albumin in two ways: firstly, the presence of albumin is essential to the detection of the platelet-stimulating activity of PAF released from rabbit basophils (Benveniste *et al.*, 1972) and for PAF release and continued PAF synthesis in human neutrophils (Ludwig *et al.*, 1985); secondly, the binding of PAF to albumin and other proteins has been demonstrated in a number of studies (Cabot *et al.*, 1985; Banks *et al.*, 1988; Clay *et al.*, 1990) and albumin greatly reduces the potency of PAF in activating platelets (Tokumura *et al.*, 1987).

Clay *et al.* (1990) have suggested that PAF binds to 4 sites on albumin with relatively high affinity ($K_d = 0.1 \mu\text{M}$). The PAF-albumin complex is the most abundant form of PAF, even when the albumin concentration is well below physiological levels (e.g. 0.25% w/v) and hence, it was concluded that the albumin complex may be the form of PAF that is active at PAF receptors (Clay *et al.*, 1990). We have made a detailed study of the influence of a range of BSA concentrations on the potency of PAF in platelets and macrophages in order to evaluate the proposal that PAF complexed to albumin is active at PAF receptors. Consideration of the impact of BSA on the potency of PAF is of importance, since BSA is most commonly used as a vehicle for pharmacological studies of PAF. BSA concentration-dependently inhibited PAF-induced aggregation of platelets and superoxide anion (O_2^-) generation by macrophages in a manner that is not consistent with PAF acting as an albumin complex.

Methods

Macrophage isolation

Male Dunkin-Hartley guinea-pigs (400–800 g) were killed by a sharp blow to the head and rapidly exsanguinated. Macrophages were isolated by peritoneal lavage: a 50 ml volume of heparinized (50 u ml^{-1}), phosphate-buffered saline was injected into the peritoneum which was gently massaged for ~1 min. The lavage fluid was aspirated and the cells were isolated by centrifugation (1000 g, 4°C, 10 min) (Stewart & Dusting, 1988).

Following isolation by centrifugation, cells were resuspended in RPMI 1640 at a concentration of 10^6 ml^{-1} , dispensed (0.5 ml) into plastic culture plates (Greiner, 24 well) and allowed to adhere for at least 2 h at 37°C. At the end of this period, non-adherent cells were removed and the remaining cells (80–90% of the total) were washed twice. This method consistently provided cells which were greater than 98% viable as assessed by trypan blue exclusion and more than 90% were macrophages as defined by non-specific esterase and Giemsa staining.

Platelet isolation

Adult rabbits (2–4 kg) were anaesthetized by intravenous administration of Saffan (alphaxalone and alphadalone). Blood was collected via a cannula placed in a carotid artery and was immediately mixed with trisodium citrate (0.38% w/v) and centrifuged at 150 g for 20 min at ambient temperature (20–24°C) (Stewart & Dusting, 1988). Prostacyclin (PGI_2) was added to a final concentration of 300 ng ml^{-1} to inhibit platelet activation during the washing procedure. The platelets were resuspended at a concentration of $2 \times 10^8 \text{ ml}^{-1}$ in Tyrode buffer (Stewart & Dusting, 1988) containing 1.8 mM Ca^{2+} with 0.0025, 0.25, 1 or 4% w/v BSA and allowed to stand at room temperature for at least 3 h before use.

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Figure 1 Comparison of measured versus predicted log EC₅₀ values for PAF-induced activation of platelets (open symbols) and macrophages (filled symbols) in experiments carried out at indicated concentrations of BSA. Predicted log EC₅₀ values were obtained as described in the text. BSA 0.0025%: platelet (○), macrophage (●); BSA 0.25%: platelet (□), macrophage (■); BSA 1.0%: platelet (▽), macrophage (▼); BSA 4.0%: platelet (◇), macrophage (◆).

Figure 2 Concentration-response relationships for PAF activation of platelet PAF receptors predicted by the model derived in the methods section based on the interaction of free PAF with the PAF receptor. BSA concentrations: 0.0025% (○), 0.25% (□); 1.0% (●); 4.0% (■).

dilution (in either Tyrode solution containing 0.25% BSA or Tyrode solution without BSA, in duplicate) a portion of the solution (100 μ l) was added to 4 ml emulsifier-safe scintillant and counted in a Packard 300C liquid scintillation spectrophotometer.

Statistical analyses

The data are presented as means and standard errors of the mean (s.e.mean) of n observations. Concentration-response lines have been analysed by linear regression of responses between 10 and 90% of the maximum response and the log EC_{50} values have been obtained by interpolation. Predicted log EC_{50} values were calculated as described in detail above. All figures were plotted using the Biosoft graphics package Fig P. Concentration-response curves were fitted to a sigmoid curve (excluding Figure 4: linear regression analyses).

Materials

All reagents and solvents used in this study were of analytical or higher grade. RPMI 1640 and FCS were obtained from CSL (Australia) and Flow Laboratories, respectively. Chemicals were obtained from the following sources: BSA, grade 5, essentially fatty acid-free; cytochrome C (horse heart type III); formyl-methionyl-leucyl-phenylalanine (FMLP); Giemsa stain; α -naphthyl acetate esterase kit; superoxide dismutase; Trydine salts (Sigma Chemical Co.); 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF) ($C_{26}H_{54}NO_7P \cdot H_2O$) (nova-biochem: Product Number, 08-74-001); NET-668 hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine-1-O-[hexadecyl-1', 2'- $^3H(N)$] (Du Pont); HEPES (N-2-hydroxy ethylpiperazine-N-2-ethane sulphonic acid (BDH chemicals); heparin (Fisons Pty. Ltd).

Results

Effect of BSA on superoxide anion generation in PAF-stimulated macrophages

To examine the possible effect(s) of BSA on O_2^- generation, concentration-response curves to PAF were constructed in the presence of increasing concentrations of BSA (0.0025–4% w/v) (Figure 3a). BSA concentration-dependently inhibited O_2^- generation in macrophages. Increasing BSA concentrations induced a rightward shift in the concentration-response curve without a suppression of the maximum response (relative to that of FMLP) indicative of competitive antagonism.

Figure 3 PAF-induced activation of guinea-pig macrophage superoxide anion (O_2^-) generation (a) and stimulation of rabbit platelet aggregation (b). Data are presented as means (s.e.mean shown by vertical bars) of a minimum of 4 observations. Concentration of BSA (solid symbols in a, open symbols in b): 0.0025% (●, ○); 0.25% (■, □); 1% (▼, ▽); 4% (●, ○).

The log EC_{50} increased from -9.81 in 0.0025% w/v BSA to -8.00 in the presence of 4% w/v BSA.

Effect of BSA on aggregation in PAF-stimulated platelets

The aggregation of rabbit platelets by PAF was examined in the presence of various concentrations of BSA (0.0025–4% w/v). Increasing concentrations of BSA shifted the concentration-response curve for PAF to the right (Figure 3b). The log EC_{50} increased progressively from -9.93 in 0.0035% w/v BSA to -8.37 in the presence of 4% w/v BSA.

Comparison of PAF potency in platelets and macrophages, with potency predicted for PAF acting as a 'free' monomer

The concentration-response curves for activation of rabbit platelets have been calculated based on the seed value of the log EC_{50} for PAF activation in the presence of 0.25% w/v BSA and the formulae (1–5, which assume that free PAF rather than that bound to BSA is the form that interacts with receptors) derived in the methods section (Figure 2). It is apparent that the predicted effect of BSA is similar to that observed (contrast with Figure 3b) with the exception of the effect of increasing BSA concentration from 0.0025% w/v to 0.25% w/v, for which the actual shift was considerably less than that predicted by the model.

A plot of the actual potency of PAF against the predicted potency of PAF (assuming that it acts as a monomer) reveals that there is a close agreement over the range of BSA concentrations between 0.25% and 4% w/v. However, as noted above, at the lowest concentration of BSA (0.0025% w/v), PAF was considerably less potent in either platelets or

Figure 4 Comparison of the potency of PAF in activation of platelet aggregation with PAF prepared in Tyrode solution containing 0.25% w/v BSA (O) and with PAF prepared in the absence of BSA (●). The platelets were washed twice in Tyrode solution to remove any residual albumin following their isolation from plasma. Data are presented as the mean and s.e.mean (vertical bars) of 3 observations.

macrophages than predicted by the model. Nevertheless, the correlation co-efficients for measured versus predicted log EC_{50} values were significant for platelets ($r^2 = 0.725$, $0.05 < P < 0.1$) and for macrophages ($r^2 = 0.914$, $P < 0.05$).

Experiments with [3H]-PAF were carried out to determine whether any losses occurred due to adsorption. PAF was prepared at a concentration of $100 \mu M$ containing either 0.25% or 0.0025% w/v BSA and 10^6 d.p.m. ml^{-1} of [3H]-PAF. Upon dilution of PAF to $10 \mu M$ with 0.25% w/v BSA, the level of radioactivity was 94,920 d.p.m. ml^{-1} , indicating that no significant losses occurred in the presence of 0.25% w/v BSA. In contrast, when PAF was diluted to $10 \mu M$ from a Tyrode solution containing 0.0025% w/v BSA into Tyrode without any BSA, the level of radioactivity was 68,711 d.p.m.

PAF stimulates platelet aggregation in the absence of BSA

The potency of PAF was compared in platelets that were incubated in the presence of BSA to that in platelets that were washed twice in the absence of albumin in order to ensure its removal. PAF was prepared in Tyrode buffer containing BSA (0.25% w/v) or in the case of platelets suspended in an albumin-free Tyrode solution, the PAF was prepared in Tyrode solution. In the absence of albumin, the apparent potency of PAF was reduced by approximately 1 log unit relative to that in the presence of 0.25% w/v BSA (Figure 4).

Discussion

It has been suggested that PAF bound to BSA is the active receptor stimulant (Clay *et al.*, 1990), and by implication, that free PAF may not be active at PAF receptors. The results of our systematic study of the effects of a wide range of BSA concentrations indicated a concentration-related inhibition of PAF-mediated responses in platelets and macrophages which is incompatible with the notion that PAF acts as a complex bound to BSA.

Studies of the actions of PAF invariably use BSA as a suspending agent, since non-specific adsorption leads to losses in more dilute solutions (Cabot *et al.*, 1985). We confirmed this observation in the present study, since PAF suspended in Tyrode solution was considerably less active

than PAF suspended in even very low albumin concentrations (0.0025% w/v). Nevertheless, this observation indicates that PAF is able to stimulate platelet PAF receptors in the absence of albumin, suggesting that albumin is not an absolute requirement for activity. Superficially, the apparent potentiation of PAF by BSA could be seen as supporting the suggestion that the PAF-albumin complex is the active species of PAF. However, it is well established that PAF is more readily solubilized in albumin-containing solutions and that losses due to adsorption and uptake into cells are greatly reduced by albumin. The dilution experiments described here further support this contention.

The reported affinity of PAF for BSA is $0.1 \mu M$ which is approximately 3 log units less than its apparent affinity for platelet receptors. Nevertheless, even the lowest concentration of BSA used in our study represents $0.4 \mu M$ ($1.6 \mu M$ binding sites) giving approximately 10^{15} binding sites per millilitre, whereas the number of PAF binding sites per platelet is 2×10^4 (Inarrea *et al.*, 1984) giving a total of 4×10^{12} PAF receptor binding sites per millilitre of platelets (suspension of $2 \times 10^8 ml^{-1}$). Thus, the excess of binding capacity of BSA compared with platelets would result in significant competition for PAF between these sites, despite the considerably higher affinity of PAF for its functional receptors. This difference in affinity is greater than indicated by apparent affinity constants determined in the presence of BSA, since these are underestimated due to the reduction in the concentration of free PAF. For example, at a total PAF concentration of 1 nM in 0.25% w/v BSA, 99.94% would be bound to BSA (as determined from equation 3). Increasing the concentration of BSA to 1% w/v would only increase the concentration of the BSA-PAF complex by 0.04%, but the concentration of free PAF would decrease from 0.6 pM to 0.2 pM. The latter decrease in free PAF concentration is the most likely explanation of the observed decrease in potency of PAF. However, it could be argued that the increase in BSA molecules not binding PAF could act as competitive antagonists of a PAF-albumin complex at the PAF receptor. Although difficult to test experimentally, this proposal seems inherently unlikely.

There was good agreement between our predicted log EC_{50} values and those obtained experimentally at concentrations of 0.25–4% w/v BSA. The log EC_{50} values obtained at 0.0025% w/v were higher than those predicted by the model. It is likely that the observed discrepancy at low BSA concentrations results from a failure of the assumption that BSA is the only significant site of loss of PAF. At low BSA concentrations, the amount of PAF bound to PAF receptors and taken up into platelets and macrophages or lost through adsorption, as was observed between concentrations of 0.25 and 0.0025% w/v BSA, may be significant in relation to the number of sites on the BSA.

It is evident that increasing the concentration of albumin reduces the concentration of free PAF, but has little effect on the concentration of BSA-bound PAF. Thus, we suggest that the decreased sensitivity to PAF of platelets or macrophages suspended in increasing concentrations of BSA (Tokumura *et al.*, 1987; present findings) indicates that free PAF, rather than BSA-complexed PAF, is active at PAF receptors. The practical implications of these findings are two fold: both binding studies and pharmacological estimates of the affinity of PAF for its receptors provide values that underestimate the true affinity of PAF; the local concentration of albumin is a prime consideration in assessing the biological activity of PAF released *in vivo*.

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