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Antagonism of the platelet activating factor-induced rise of the intracellular calcium ion concentration of U937 cells

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1 U937 cells are a continuous line of human cells of committed monocytic origin which serve as a useful model for studying human monocytic function. The present study investigated the effect of platelet-activating factor (Paf) on intracellular free calcium ion concentration ($[Ca^{2+}]_i$) in U937 cells using the calcium fluorescent probe fura-2.

2 The naturally-occurring stereoisomer (**R**)-Paf (0.01–300 nM) and the stable, less hydrolysable racemic Paf analogue PR1501 (10 nM–3 μM) produced dose-related and rapid elevations of 100–1200 nM $[Ca^{2+}]_i$ above a basal value of 135 ± 9 nM ($n = 22$).

3 The unnatural stereoisomer (**S**)-Paf and the natural stereoisomer lyso-(**R**)-Paf had no effect on basal $[Ca^{2+}]_i$ at 30 μM, approximately 100,000 times the concentration found to be the threshold concentration to elicit a response to (**R**)-Paf.

4 Leukotriene B_4 (LTB_4) also induced increases in $[Ca^{2+}]_i$ in the concentration range 28.5 nM–2.85 μM but the responses were smaller and of shorter duration than those induced by Paf.

5 Five compounds, WEB 2086, Ro 19-3704, L-652,731, BN 52021, and CV 3988, inhibited sub-optimal Paf (10 nM)-induced increase in $[Ca^{2+}]_i$ with IC_{50}s of 48 ± 2, 118 ± 33, 318 ± 131, 340 ± 205 and 2320 ± 183 nM respectively. All five compounds have previously been reported as specific Paf receptor antagonists, at least with respect to platelets.

6 The above compounds at 10 μM had no effect upon the increased $[Ca^{2+}]_i$ induced by either LTB_4 or the calcium ionophore, ionomycin.

7 These results suggest that U937 cells respond to Paf at least with respect to elevated $[Ca^{2+}]_i$ as measured by fura-2 and that these cells may well possess a Paf receptor as suggested by the action of specific antagonists and the stereoselectivity observed with Paf.

Introduction

U937 cells are a continuous line of human cells of committed monocytic origin which are derived from the pleural exudate of a patient with diffuse histiocytic lymphoma (Sundstrom & Nilsson, 1976). They represent immature monocytic cells and upon stimulation to differentiate by appropriate stimuli, undergo morphological and functional maturation similar to that described for normal monocytic cells. U937 cells have some macrophage characteristics including esterase, surface Fc and C3 receptors, and the capacity for phagocytosis, lysozyme synthesis and antibody-dependent cell-mediated cytotoxicity (Harris & Ralph, 1985). Furthermore, like human monocyte-macrophages, U937 cells are a source of monokines such as interleukin-1 (Palacios *et al.*, 1982; Barak *et al.*, 1986) and can, under appropriate conditions, be stimulated to produce and release prostaglandin E_2 (Cobb *et al.*, 1983; Roux-Lombard *et al.*, 1986). Therefore, they serve as a useful model for studying human monocyte maturation and function.

In a previous study, we showed that platelet-activating factor (Paf, 1-0-octadecyl-2-0-acetyl-sn-glycero-3-phosphoryl-choline) a lipid mediator which is produced by and activates a range of inflammatory cells, (reviewed by Braquet *et al.*, 1987) induced IL-1-like activity from human monocyte-macrophage cultures (Barrett *et al.*, 1987). Although the actions of Paf on platelets and neutrophils are well documented (Chignard *et al.*, 1985; Westwick &

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Poll, 1987) the actions of Paf on monocyte-macrophages, particularly of human origin, are less well known.

Platelet-activating factor has been shown to activate guinea-pig peritoneal macrophages in terms of increased glucose consumption (Hayashi *et al.*, 1985a,b), superoxide generation (Hartung *et al.*, 1983), chemiluminescence (Lambrecht & Parnham, 1986) and enhanced prostaglandin E_2 (PGE_2) and thromboxane B_2 (TxB_2) release (Hartung, 1983). More recently, Paf has been shown to elevate $[Ca^{2+}]_i$ in guinea-pig peritoneal macrophages (Conrad & Rink, 1986) and in U937 cells (Maudsley & Morris, 1987). Despite these functional events evoked by Paf, there has been a report that there is no specific Paf receptor on guinea-pig macrophage membranes (Hwang *et al.*, 1983).

The purpose of this study was to characterize the nature of the Paf receptor present on U937 cells by measuring an early post-receptor event, namely elevation of $[Ca^{2+}]_i$ by using U937 cells loaded with the fluorescent probe fura-2 (Grynkiewicz *et al.*, 1985). Receptor characterization was aided by the use of natural and unnatural enantiomers of Paf, a racemic stable analogue, and five compounds that have been described as specific Paf receptor antagonists, at least with respect to platelet activation.

Methods

Cell line

U937 cells were grown in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated foetal calf serum (HIFCS) and 100 units ml^{-1} penicillin/streptomycin (Gibco) at 37°C in a humidified atmosphere at 5% CO_2.

Loading with fura-2

U937 cells were suspended at 10^7 cells ml^{-1} in RPMI-1640 medium supplemented with 10% HIFCS and incubated for 30 min at 37°C with 2.5 μM fura-2 acetoxymethyl ester. After incubation, the cells were washed three times in 20 ml of calcium-free, HEPES buffered, Tyrode solution (composition, mM: HEPES 10, NaCl 145, KCl 5, $MgCl_2$ 1, Na_2HPO_4 0.5, glucose 5.5 and 0.25% bovine serum albumin, pH 7.4, 37°C) and finally re-suspended at 2×10^6 cells ml^{-1} in HEPES buffer.

Intracellular free calcium

Aliquots of cells (1 ml) suspended in HEPES buffered Tyrode solution were dispensed into disposable cuvettes and the external Ca^{2+} concentration ($[Ca^{2+}]_o$) adjusted to 1 mM with $CaCl_2$. Cuvettes were transferred to an Aminco Bowman spectrophotofluorimeter (excitation λ 339 nm, emission λ 500 nm–4 nm slit width, fitted with thermostatted cuvette compartment holder with stirring attachment) for fluorescence reading at 37°C. Leakage of fura-2 from the U937 cells was shown to be small as determined by the quench in fluorescence signal produced by the addition of 1 mM Ni^{2+}. The $[Ca^{2+}]_i$ of the U937 cells was determined by the chelation method and formula as described in detail by Pollock *et al.* (1986). The F_{max} was determined by lysing the cells with 40 μM digitonin in the presence of 1 mM Ca^{2+}. The F_{min} was determined by adjusting the pH of the lysed cells to 8.5 with 20 mM Tris base followed by the addition of 10 mM EGTA. Cells were allowed to equilibrate at 37°C for 2 min before agonists or vehicle were added directly to the cell-containing cuvettes. Potential antagonists or vehicles were added 1 min before the agonist.

Materials

Compounds used and the source of their supply were as follows: octadecyl-(R)-Paf and octadecyl-lyso-(R)-Paf, Bachem, Switzerland; octadecyl-(S)-Paf, Hoffman-La Roche, Switzerland; PR1501 (racemic 1-0-octadecyl-2-acetamido-2-deoxy-glycero-3-phosphocholine), Leo Pharmaceuticals, Denmark; L-652,731 ((±)-2,5-*bis*-(3,4,5-trimethoxyphenyl) tetrahydrofuran), the Merck Institute, Rahway, USA; BN 52021 (3-(1,1-dimethylethyl)hexahydro-1,4,7b-trihydroxy-8-methyl-9H-1,7-(epoxymethano)-1H,6H-cyclopenta[C]furo (2,3b)(3',2':3,4)cyclopenta (1,2-d)furan-5,9,12(4H)-trione), Institut Henri-Beaufour, Paris, France; CV 3988 [(RS)-2-methoxy-3-(octadecylcarbamoyloxy)propyl-2-(3-thiazalium)ethyl phosphate], Takeda Chemical Industries, Osaka, Japan; WEB 2086 (3-[4-(2-chlorophenyl-9-methyl-6H-thienol(3,2-f)(1,2,4)triazolo-(4,3-a)(1,4)-diazepin-2-yl]-1-(4- morpholinyl)-1-propanone), Boehringer Ingelheim KG; Ro 19-3704, (3-(4-[(R)-2-[(methoxycarbonyl)oxy]-3-[(octadecylcarbamoyl)-oxy]propoxy]butyl)thiazolium iodide), Hoffman-La Roche; fura-2 acetoxymethyl ester, Molecular Probes, Eugene, Oregon, U.S.A.; bovine serum albumin was purchased from Sigma, Poole, Dorset.

Results

Changes in $[Ca^{2+}]_i$ in response to Paf, PR1501 and LTB_4

Addition of either (R)-Paf (0.1–300 nM) or PR 1501 (10 nM – 3 μM) to fura-2-loaded U937 cells suspended in HEPES buffered Tyrode solution containing 1 mM $[Ca^{2+}]_o$ produced a rapid elevation of $[Ca^{2+}]_i$

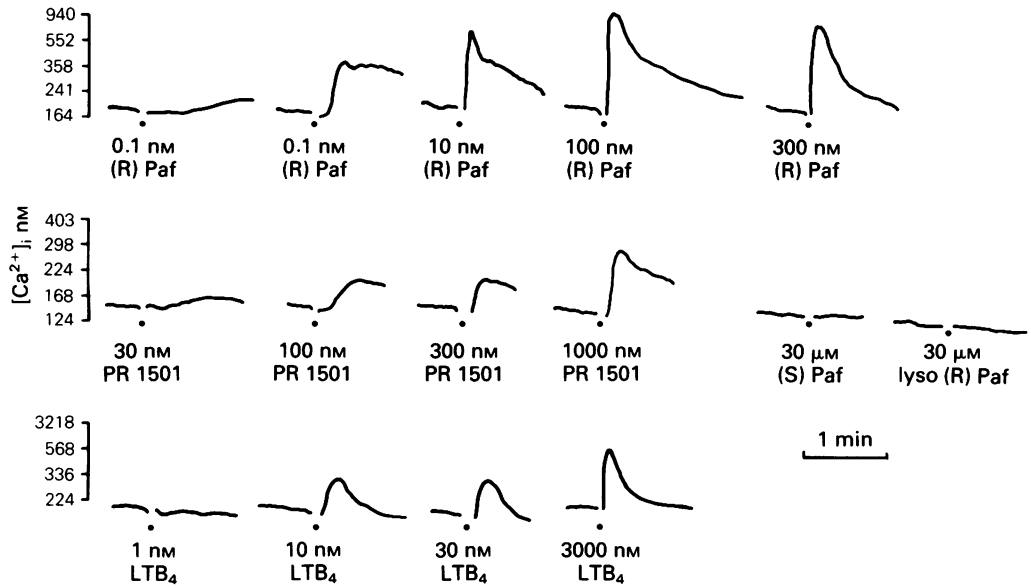

Figure 1 Effect of (R)-Paf (platelet activating factor), PR1501, (S)-Paf, lyso-(R)-Paf and leukotriene B_4 (LTB_4) on U937 cell $[Ca^{2+}]_i$. The trace is a recording of fluorescent output of the fura-2-loaded U937 cells. The non-linear vertical scale is the result of transforming fluorescent output to U937 $[Ca^{2+}]_i$. The traces were obtained from one experiment, but are representative of five other experiments.

above basal values of 135 ± 9 nM ($n = 22$) to approximately 200–1200 nM depending upon dose (Figure 1). As can be seen from the traces of fluorescent output (Figure 1), increasing the dose of (R)-Paf reduces the delay in response and accelerates the rate of elevation and increases the peak calcium signal attained. Furthermore, the decline of the calcium signal, especially with the higher doses of (R)-Paf, occurs in two stages, characterised by a rapid decline followed by a protracted decline to basal values. With all doses of (R)-Paf and PR1501 examined, the elevated $[Ca^{2+}]_i$ has returned to basal values within 3 min. The Paf analogue PR1501 was approximately

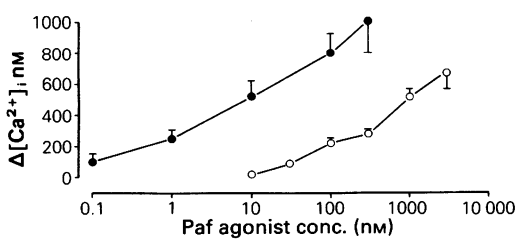

Figure 2 Dose-response curves of (R)-Paf (●) and PR1501 (○)-induced elevation of $[Ca^{2+}]_i$. $\Delta[Ca^{2+}]_i$ is the value obtained by subtracting basal pre-agonist value from the peak post-agonist value. Each point (●, ○) represents the mean of determinations performed in 4 to 6 experiments; vertical lines show s.e. mean.

100 times less potent than (R)-Paf in its ability to induce an elevation of $[Ca^{2+}]_i$ (Figures 1 and 2). In marked contrast, neither (S)-Paf, the unnatural enantiomer of (R)-Paf, nor the precursor/metabolite lyso-(R)-Paf produced any effect upon $[Ca^{2+}]_i$ at 30 μM.

Leukotriene B_4 (28.5 nM–2.85 μM) also produced a rapid concentration-dependent elevation in $[Ca^{2+}]_i$ (Figure 1). The LTB_4-induced $[Ca^{2+}]_i$ elevation differed markedly from that induced by (R)-Paf, since the responses were generally much faster in reaching maximum and of shorter duration, the entire response being complete in around 20–30 s, irrespective of concentration. Maximal elevation of $[Ca^{2+}]_i$ (400 nM) was achieved with 2.85 μM LTB_4 whilst a similar elevation was achieved with approximately 10 nM Paf.

Inhibition of the elevation of $[Ca^{2+}]_i$ by Paf-receptor antagonists

Five Paf-receptor antagonists, namely WEB 2086 (10–300 nM), Ro 19-3704 (10 nM–10 μM), BN 52021 (10 nM–30 μM) L-652,731 (10 nM–100 μM) and CV 3988 (10 nM–100 μM) were effective inhibitors of suboptimal Paf (10 nM)-induced elevation of $[Ca^{2+}]_i$. The mean percentage inhibition of 10 nM Paf-induced elevation of $[Ca^{2+}]_i$ produced by the Paf receptor antagonists is shown in Figures 3a and b. IC_{50} values for all five antagonists are shown in

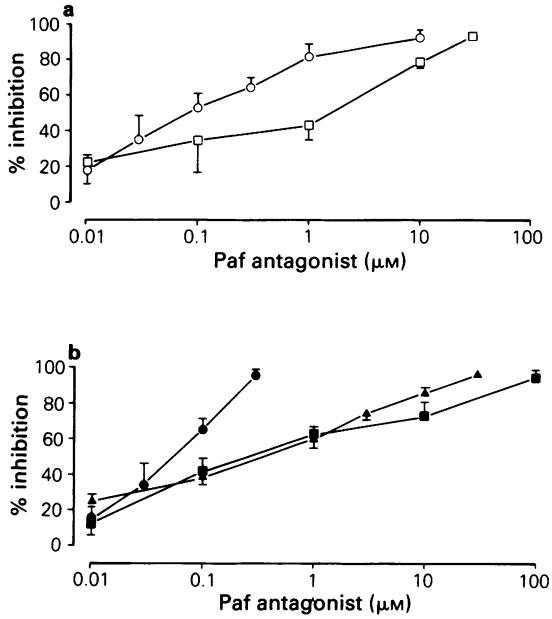

Figure 3 (a) Dose-response curves of inhibition by Paf (platelet activating factor) antagonists of 10 nM Paf-induced elevation of $[Ca^{2+}]_i$: Ro 19-3704 (○) and CV 3988 (□). (b) Dose-response curves of inhibition by Paf antagonists of 10 nM Paf-induced elevation of $[Ca^{2+}]_i$: WEB 2086 (●), BN 52021 (▲) and L-652,731 (■). Each point represents the mean of determinations performed in 5 to 6 experiments; vertical lines show s.e. mean.

Table 1. The most potent Paf antagonist in this system was WEB 2086 and it was also found to be a potent inhibitor of PR1501 (data not shown).

The calcium ionophore, ionomycin, provides a pharmacological tool with which to elevate $[Ca^{2+}]_i$ whilst bypassing receptor-operated systems. We found that 2 μM ionomycin evoked micromolar changes in $[Ca^{2+}]_i$ above basal levels, with the response being fast and much prolonged, far more so than either Paf or LTB_4 responses (data not shown).

The Paf-receptor antagonists when examined at a concentration of 10 μM were unable to modify either LTB_4 (300 nM)- or ionomycin (2 μM)-induced elevation of $[Ca^{2+}]_i$ (data not shown).

Discussion

The results presented here show clearly that (R)-Paf induces an elevation of $[Ca^{2+}]_i$ in U937 cells, and support similar results recently obtained by Maudsley & Morris (1987). Furthermore, this effect can be specifically inhibited by five compounds, namely WEB 2086, Ro 19-3704, L-652,731, BN 52021, and CV 3988 which have been shown to be antagonists of Paf with respect to platelet activation or [^{3}H]-Paf binding to platelets (Terashita *et al.*, 1983; Burri *et al.*, 1985; Hwang *et al.*, 1985; Braquet *et al.*, 1985; Nunez *et al.*, 1986; Casals-Stenzel *et al.*, 1986). With the U937 cells, the inhibitory actions appear to be specific to Paf since the antagonists at high concentrations did not affect the responses seen with LTB_4 or ionomycin. Furthermore, the lack of effect of the Paf-receptor antagonists on both LTB_4- and ionomycin-induced calcium mobilisation, indicates that these compounds do not modify transmembrane coupling mechanisms involved in calcium mobilisation in these cells. The stable non-hydrolysable analogues of Paf, e.g. PR1501, appear to be active in the same concentration-range as is seen in platelets (Hadvary *et al.*, 1983), and the elevated $[Ca^{2+}]_i$ induced by PR1501 could also be inhibited by concentrations of WEB 2086 that effectively wipe out Paf responses.

The unnatural stereoisomer of (R)-Paf, the (S) form, was found to be inactive in this system, even at 100,000 times the concentration found to be the threshold concentration to elicit a response to the naturally occurring stereoisomer (R)-Paf. This is similar to the activity seen for both the (R) and (S) forms of Paf with platelets (Hadvary & Baumgartner, 1983). Although a clear demonstration of receptors for Paf on U937 cells has not been shown here

Table 1 Effect of platelet Paf receptor antagonist on Paf-induced elevation of $[Ca^{2+}]_i$ of U937 cells

Compound	IC_{50} (nM)	*P**	*Relative potency*
WEB 2086	48 ± 2	*	1
Ro 19-3704	118 ± 33		2.5
BN 52021	340 ± 205	**	7.1
L-652,731	318 ± 131	**	66
CV 3988	2320 ± 183	**	48.3

For details of compounds see *Materials* section.
* $P < 0.05$ ** $P < 0.005$ (Student's t test).

in terms of binding studies, a number of lines of evidence support the concept of specific receptors for Paf. These include the low concentrations required for target cell stimulation as measured in terms of elevated $[Ca^{2+}]_i$, the relatively specific inhibitory action of the Paf receptor antagonists, the stereospecificity of the effect and the fact that lyso-(R)-Paf, which is both the precursor and metabolite of (R)-Paf, is inactive in this system.

Platelet-activating factor receptor sites have been detected on the plasma membranes of human and rabbit platelets (Valone *et al.*, 1982; Hwang *et al.*, 1983; Kloprogge & Akkerman, 1984; Tuffin *et al.*, 1985; Cavero *et al.*, 1987), bovine and guinea-pig polymorphonuclear leucocytes (Hwang *et al.*, 1983; Valone & Goetzl, 1983), rabbit ileum, guinea-pig ileum and lung (Hwang *et al.*, 1983). There are conflicting reports as to whether or not there are single affinity binding sites (Kloprogge & Akkerman, 1984; Hwang *et al.*, 1985; Cavero *et al.*, 1987) or two binding sites of high and low affinity (Valone *et al.*, 1982; Valone & Goetzl, 1983; Tuffin *et al.*, 1985). We are now examining [^{3}H]-Paf binding to U937 cells so that we may further characterize the Paf receptor present on these cells.

It would appear that signal transduction following Paf-receptor binding intimately involves guanyl nucleotide regulatory proteins. (Haslam *et al.*, 1985; Lad *et al.*, 1985; Hwang *et al.*, 1986). An investigation into the mechanism of stimulus-response coupling in U937 cells, especially with respect to the guanyl nucleotide regulatory proteins and polyphosphoinositol metabolism (Berridge & Irvine, 1984), may elucidate the mechanism by which Paf interacts with plasma membrane receptors to induce calcium mobilisation.

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