Volume 243, number 1, 41–46 FEB 06684 January 1989

Characterization of platelet-activating factor-induced elevation of cytosolic free calcium concentration in eosinophils

Claus Kroegel, Robert Pleass*, Tatsuo Yukawa, K. Fan Chung, John Westwick* and Peter J. Barnes

Department of Thoracic Medicine, National Heart and Lung Institute, Brompton Hospital, University of London, Dovehouse Street, London SW6 LY6 and **Department of Pharmacology, The Hunterian Institute, Royal College of Surgeons, Lincoln's Inn Fields, London WC2A 3PN, England*

Received 8 November 1988

In order to evaluate the role of calcium in the activation processes in eosinophils induced by platelet-activating factor (PAF), we investigated the changes in free cytoplasmatic Ca^{2+} concentration using fura-2. PAF causes a rapid and transitory rise of the intracellular free calcium ion concentration ($[Ca^{2+}]_i$) in purified guinea pig eosinophils of approx. 1000 nM above a basal level of 120.7 ± 36.5 nM ($n = 10$). The effect was dose-related with a maximum rise at 1000 nM PAF and an EC_{50} of 17.4 nM and specifically inhibited by the PAF antagonist WEB 2086 with an IC_{50} of 95.5 nM. WEB 2086 did not affect either the leukotriene B_4- or the fMet-Leu-Phe-induced elevation of $[Ca^{2+}]_i$. The response to PAF was dependent on external Ca^{2+} as it was significantly inhibited by EGTA ($85.6 \pm 5.4\%$) and Ni^{2+} ($95.8 \pm 2.1\%$) but not by the dihydropyridine antagonist nimodipine. We conclude that Ca^{2+} entry via receptor-operated Ca^{2+} channels may be involved in PAF-induced degranulation of eosinophils.

Ca^{2+} concentration, intracellular; Platelet-activating factor; Platelet-activating factor antagonist WEB 2086

1. INTRODUCTION

Platelet-activating factor (PAF) is now considered to be a major inflammatory mediator in bronchial asthma [1]. Asthma is characterized by infiltration of the airway wall by eosinophils. Eosinophil accumulation in lung tissue is stimulated by PAF [2]. Eosinophils in turn may be involved in the development of bronchial hyperresponsiveness and epithelial damage in asthmatic airways by releasing their basic proteins such as major basic protein, eosinophil cationic protein and eosinophil peroxidase [3].

Eosinophils are stimulated by a number of agents including the calcium ionophore, formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe), and phagocytosable particles [3]. In addition, PAF shows potent eosinophil chemotactic activity [4] and stimulates the generation of leukotriene C_4 in these cells [5]. Furthermore, we have demonstrated that PAF releases eosinophil peroxidase (EPO) and other granular contents from human and guinea pig eosinophils [6]. This observation may be of particular pathogenetical interest since it links PAF with a putative effector cell in asthma.

In order to investigate the mechanism of PAF-induced eosinophil activation we have studied the change in intracellular calcium ion concentration ($[Ca^{2+}]_i$) of purified guinea pig eosinophils.

2. MATERIALS AND METHODS

2.1. *Materials*

Hanks balanced salt solution (HBSS) was purchased from Flow Laboratories Ltd (Richmansworth, England), Percoll was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden), and octadecyl-PAF and octadecyl-lyso-PAF were obtained from Bachem (Switzerland). WEB 2086 was kindly

Correspondence address: P.J. Barnes, Department of Thoracic Medicine, National Heart and Lung Institute, Brompton Hospital, Dovehouse Street, London SW3 LY6, England

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

donated by Boehringer Ingelheim (Ingelheim, FRG). Triton X-100 was purchased from BDH Ltd (Poole, England). Leukotriene B_4 (LTB_4), *N*-formylmethionyl-leucyl-phenylalanine, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), polymyxin B sulfate, EGTA and digitonin were from Sigma (Poole, England) and fura-2 acetoxymethyl ester from Molecular Probes (Eugene, OR, USA).

2.2. *Preparation of eosinophils*

Eosinophils were obtained from polymyxin B-treated male guinea pigs (650–1000 g) by weekly peritoneal lavage [7]. The cells were purified using a discontinuous gradient of isomolar Percoll solutions (1.100, 1.090, 1.085, 1.080, and 1.070 g/ml) as previously described [8]. Eosinophils with a purity of >95% and a viability >99% were found in fractions 3, 4, 5 and 6. The fractions were pooled and washed three times in HBSS buffer.

2.3. *Measurements of $[Ca^{2+}]_i$*

The cells were then incubated for 30 min at 37°C (10^7 cells/ml) with 2.5 μM fura-2 as described [9]. After incubation, eosinophils were washed three times in calcium-free, Hepes buffered Tyrode solution and resuspended to a final concentration of 1.5×10^6 cells/ml. 2 ml of the cell suspension were dispensed into disposable cuvettes and the external Ca^{2+} concentration ($[Ca^{2+}]_o$) adjusted to 1 mM with CaCl. Cuvettes were transferred to a Bowman spectrophoto-fluorimeter (excitation 330 nm, emission 500 nm: 4 nm slit width), fitted with a stirring attachment for fluorescence reading at 37°C. Leakage of fura-2 from eosinophils was shown to be small as determined by the degree of quench in fluorescence signal produced by the addition of 1 mM Ni^{2+}. The intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by the chelation method and formula [10]. F_{max} was measured by lysing with 40 μm digitonin in the presence of 1 mM Ca^{2+}. F_{min} was determined by adjusting the pH of the lysed cells to 8.5 with 20 μl 2 M NaOH followed by the addition of 10 mM EGTA. Cells were allowed to equilibrate at 37°C for 2 min before the agonist or vehicle was added directly to the cell-containing cuvettes. WEB 2086 and the vehicle were added 1 min before the agonist.

2.4. *Statistics*

Negative log EC_{50} values from each concentration-response curve were derived by linear regression analysis of percentage of the maximum $[Ca^{2+}]_i$ for the agonist versus log concentration at concentrations immediately above and below the 50% response level. For each stimulus, experiments were performed at least three times in duplicate or triplicate by using cell preparations from different animals on different days. Results were expressed as mean ± SE and were analysed by Student's *t*-test.

3. RESULTS

Addition of PAF to eosinophils loaded with

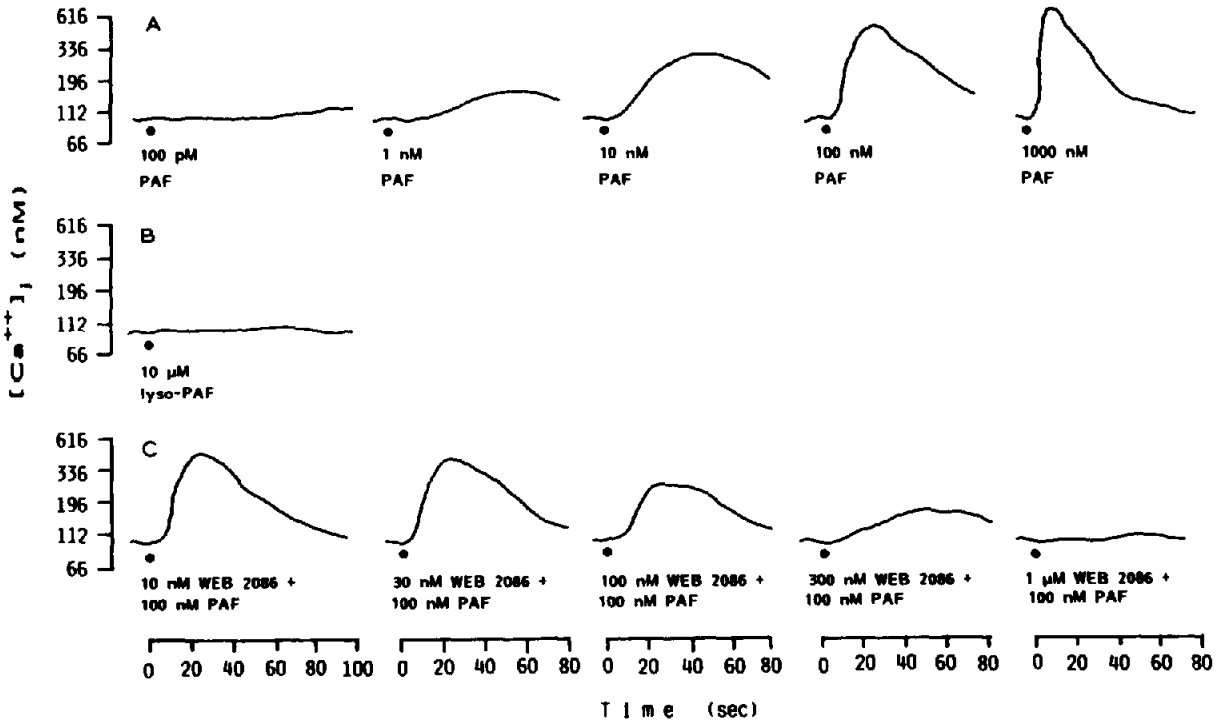

Fig.1. Effect of PAF (A), lyso-PAF (B) and WEB 2086 (C) on intracellular free calcium ion concentration ($[Ca^{2+}]_i$) in eosinophils. The trace is a recording of fluorescent output of fura-2-loaded guinea pig eosinophils (3×10^6 cells/sample). The non-linear vertical scale is the result of transforming fluorescent output to eosinophil $[Ca^{2+}]_i$. The traces were obtained from one experiment, but are representative of four other experiments.

fura-2 produced a rapid elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$) above basal values of 120.7 ± 36.5 nM (n = 10) up to approx. 1000 nM. The effect of PAF was concentration-dependent with a maximal effect seen at 1000 nM and a mean EC_{50} of 17.6 nM (calculated from five independent experiments). Increasing PAF concentrations also reduced the time to reach both the onset and the maximum of response as well as the time interval between them (fig.1A). Lyso-PAF was ineffective at concentrations up to 10 μM (fig.1B). Preincubation with specific PAF receptor antagonist WEB 2086 [13] for 1 min inhibited the elevation of $[Ca^{2+}]_i$ induced by PAF (100 nM) in a dose-dependent manner with a corresponding IC_{50} of 59.5 nM (fig.1C).

LTB_4 and fMet-Leu-Phe also increased $[Ca^{2+}]_i$ in eosinophils. On a molar basis, PAF and LTB_4 were equipotent whereas for fMet-Leu-Phe higher concentrations (1–100 μM) were needed to elicit a rise of $[Ca^{2+}]_i$ in eosinophils. In addition, LTB_4 and PAF showed a similar time course, whereas FMLP was slower in onset and longer lasting (fig.2). After an initial exposure to PAF eosinophils were unresponsive to a second application of PAF, indicating homologous desensitization (fig.3A). In contrast, an initial exposure to PAF did not affect the responses produced by a subsequent application of LTB_4 or fMet-Leu-Phe (fig.3).

When eosinophils were stimulated with PAF in a Ca^{2+}-free buffer in the presence of 5 mM EGTA, $[Ca^{2+}]_i$ still displayed a slight rise (fig.4), but it was greatly diminished compared with the rise seen at 1 mM Ca^{2+}. The mean inhibition for 100 pM–1 μM PAF was 85.6 ± 5.4 ($p < 0.05$). Replacement of 1 mM $[Ca^{2+}]_e$ with 1 mM Ni^{2+} produced an instantaneous slight quench of the signal indicating a small leakage of intracellular fura-2. The initial quench is followed by a signifi-

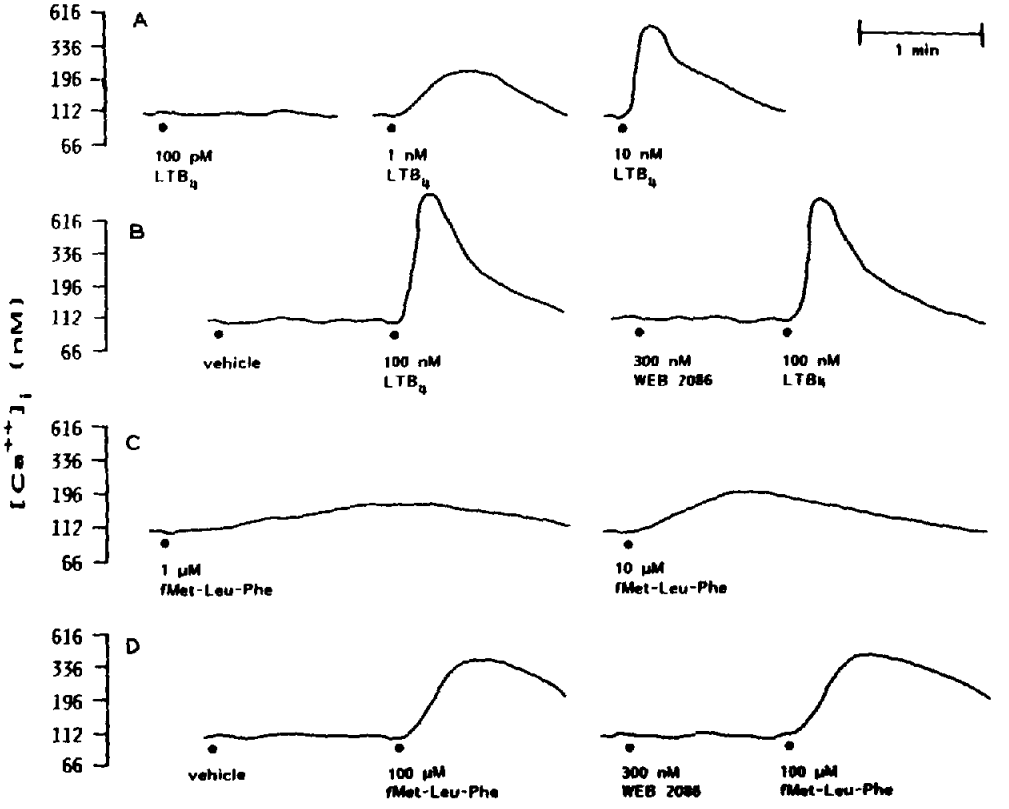

Fig.2. Effect of leukotriene B_4 and fMet-Leu-Phe on intracellular free calcium concentration in eosinophils. Panels B and D demonstrate the influence of WEB 2086 on both stimuli. Details as described in fig.1.

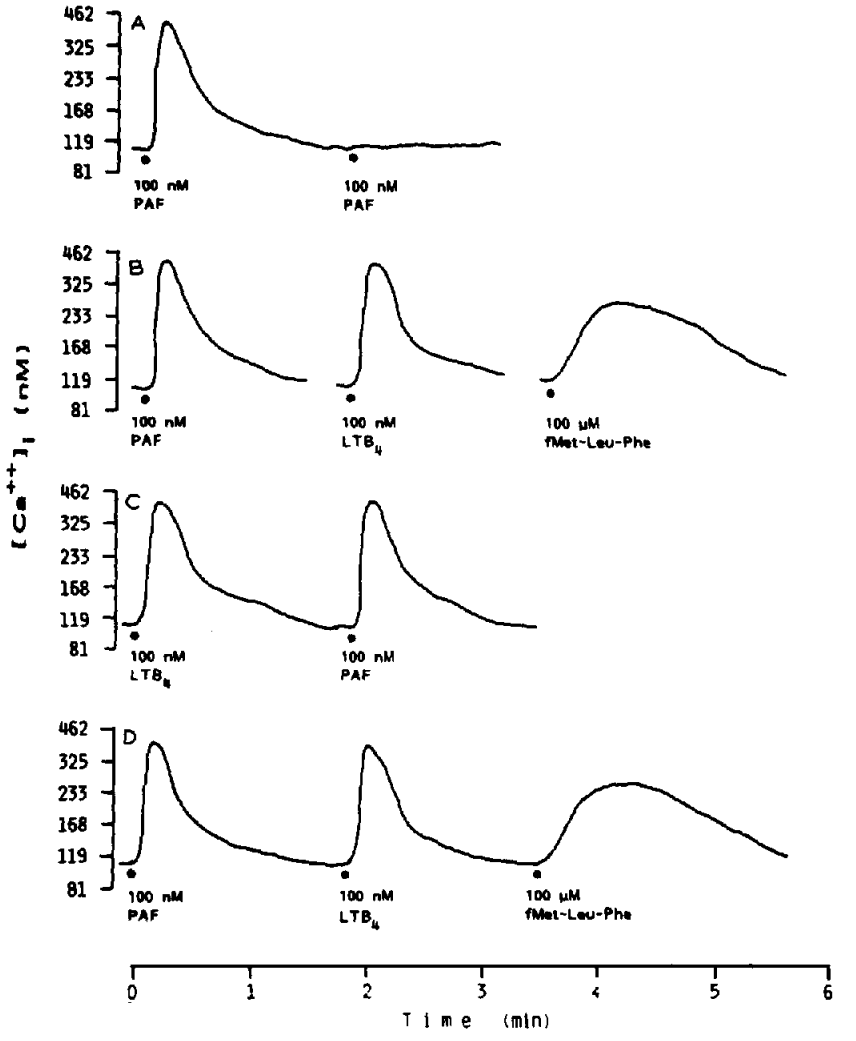

Fig.3. Comparison of repeated stimulation of the same eosinophil sample by PAF (A), leukotriene B_4 (C), or fMet-Leu-Phe (D) with their effect on independent cell samples (B). Details as described in fig.1.

cant prolongation of the response time and a marked decrease in the peak elevation of $[Ca^{2+}]_i$ upon stimulation of PAF. Ni^{2+}, which are known to block calcium-translocating pores [12], produced an almost complete inhibition (95.8 ± 2.1; $p > 0.05\%$) of elevation in $[Ca^{2+}]_i$ elicited by PAF at concentrations ranging from 100 pM to 1 μM. These data suggest that the increase in $[Ca^{2+}]_i$ induced by PAF arose mainly from an influx of exogenous Ca^{2+}. Moreover, preincubation of eosinophils for 10 min with the dihydropyridine antagonist nimodipine up to concentrations of 10 μM did not affect the elevation of $[Ca^{2+}]_i$ by PAF, indicating that dihydropyridine-sensitive Ca^{2+} channels are not involved.

Finally, exposure of eosinophils to 1 mM Mn^{2+} led to an instantaneous small sharp drop followed by a decline in fluorescence. As for Ni^{2+}, the slight drop in fluorescence upon addition of Mn^{2+} reflects the immediate quenching of leaked extracellular fura-2 while the subsequent decline indicates a moderate influx of Mn^{2+} into the cells displacing Ca^{2+} from the intracellular fura-2 and quenching the fluorescence signal. In contrast to

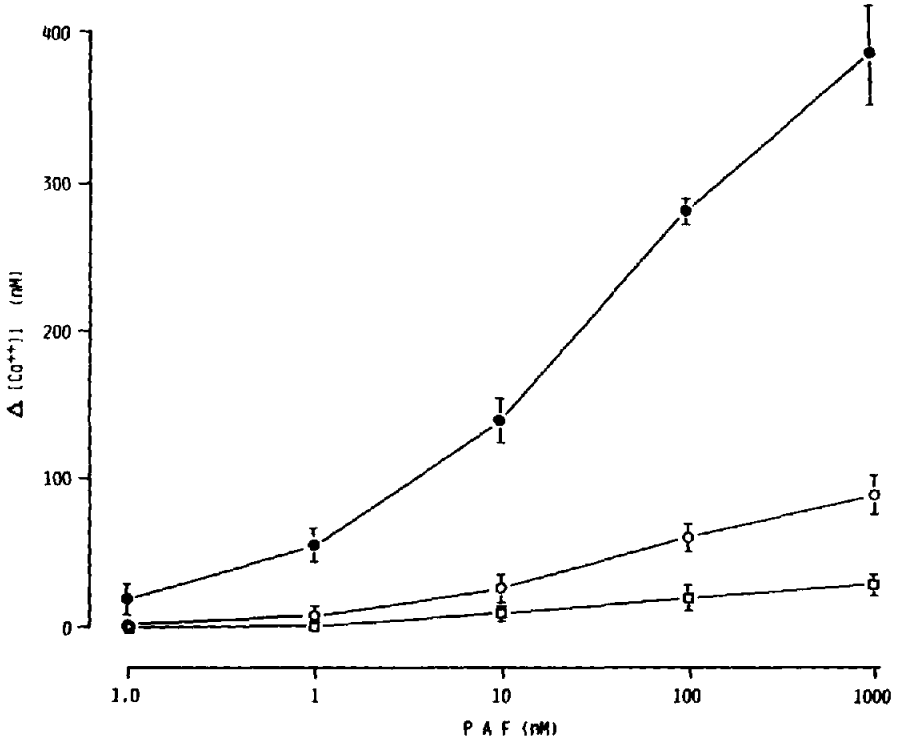

Fig.4. Dose-response curve of PAF-induced $[Ca^{2+}]_i$ in the presence of 1 mM Ca^{2+} (●), 5 mM EGTA (○), and 1 mM Ni^{2+} (□). $[Ca^{2+}]_i$ is the value obtained by subtracting basal pre-agonist value from the peak post-agonist value. Data are expressed as mean ± SE of 9 determinations from three independent experiments.

observations in platelets [21,22], PAF did not stimulate Mn^{2+} entry in eosinophils.

4. DISCUSSION

The results presented here demonstrate that PAF induces a rapid and transient increase of $[Ca^{2+}]_i$ in guinea pig eosinophils in a dose-dependent manner with a maximum effect at 1000 nM and a mean EC_{50} of 17.6 nM. Furthermore, the cellular response to PAF was inhibited in the presence of WEB 2086 (EC_{50} 59.5 nM). WEB 2086 has been shown to be a receptor antagonist of PAF with respect to activation and binding to platelets [13], neutrophils [14], and U937 cells [9]. With eosinophils, its inhibitory action appears to be specific to PAF since the agonist at high concentrations (1 μM) did not affect the response seen with leukotriene B_4 or fMet-Leu-Phe. These results suggest that PAF acts on guinea pig eosinophils through a specific, receptor-mediated mechanism.

There are at least two functionally distinct pathways by which PAF increases $[Ca^{2+}]_i$: release of calcium from intracellular stores and calcium influx through plasma membrane pores. The importance of extracellular Ca^{2+} ($[Ca^{2+}]_e$) in response to PAF has been demonstrated in platelets [15], macrophages [17], and U937 cells [16]. The effect of PAF on eosinophils also appears to involve a major influx of $[Ca^{2+}]_e$ through receptor-mediated pores. PAF-induced rise of $[Ca^{2+}]_i$ in eosinophils was greatly diminished in the presence of submicromolar $[Ca^{2+}]_o$, EGTA, or Ni^{2+} (fig.4). Moreover, dehydropyridine-sensitive Ca^{2+} channels do not appear to be involved in the ion influx since the dihydropyridine antagonist nimodipine has no effect on the PAF-induced elevation of $[Ca^{2+}]_i$.

When eosinophils were sequentially exposed to PAF, then leukotriene B_4 and then fMet-Leu-Phe, the cells produced a full response to each stimulus. If the intracellular calcium stores are depleted dur-

ing the first stimulation, a diminished response to subsequent stimuli might occur. However, this was not the case, since repeated application of different agonists produced a full response. The results support the concept that receptor-operated influx of Ca^{2+} is necessary to obtain a maximal elevation of $[Ca^{2+}]_i$. However, our studies also show a small but consistent elevation of $[Ca^{2+}]_i$ elicited by PAF both in the absence of Ca^{2+} and in the presence of Ni^{2+}. These results also suggest that PAF, in addition to the stimulated influx, causes release of some Ca^{2+} from intracellular stores as previously described in platelets, macrophages, and U937 [15–17]. Furthermore, an apparent increase in membrane permeability to Mn^{2+}, as seen in platelets in response to PAF [21,22], was not observed in eosinophils.

PAF at concentrations from 10 nM to 10 μM causes eosinophil chemotactic locomotion [4] and eosinophil adhesion to endothelial cells [18]. In addition, PAF at concentrations of 10 μM induces leukotriene C_4 synthesis in human eosinophils [5]. Furthermore, we have demonstrated that PAF is a potent degranulating agent for human and guinea pig eosinophils at concentrations ranging from 10 μM to 0.1 nM [6]. The biochemical mechanism, however, by which PAF elicits these responses is not yet fully understood. The concentrations of PAF needed to elicit the functional effects and to increase $[Ca^{2+}]_i$ in eosinophils are similar with an effect starting at 10 pM and reaching a maximum at 10 μM, indicating that the transduction signal generated by PAF-receptors in eosinophils may involve an elevation of $[Ca^{2+}]_i$. A similar relation between the PAF-induced cellular response and $[Ca^{2+}]_i$ have been described for peritoneal macrophages from mice [17] and guinea pig neutrophils [19] as well as for platelets [20].

In summary, our results confirm that the eosinophil is a target cell for PAF. Furthermore, we conclude that the response of eosinophils to PAF appears to be receptor mediated and that the rise of $[Ca^{2+}]_i$ represents an early event in eosinophil cell activation. Finally, the intracellular elevation of the calcium concentration results mainly from an influx of extracellular Ca^{2+} via receptor-operated rather than from voltage-dependent Ca^{2+} channels. Since both the eosinophil and PAF may play a crucial role in the pathogenesis of asthma our findings may be of particular pathophysiological relevance.

REFERENCES

[1] Barnes, P.J., Chung, K.F. and Page, C.P. (1988) J. Allergy Clin. Immunol. 81, 919–934.
[2] Lellouch-Tubiana, A., Lefort, J., Pirotzky, E., Vargaftig, B.B. and Pfister, A. (1985) Br. J. Exp. Pathol. 66, 345–355.
[3] Gleich, J.G. and Adolphson, C.R. (1986) Adv. Immunol. 39, 177–253.
[4] Wardlaw, A.J., Moqbel, R., Cromwell, O. and Kay, A.B. (1986) J. Clin. Invest. 78, 1701–1706.
[5] Bruynzeel, P.L.B., Kok, P.T.B., Haemelink, M.L., Kijne, A.M. and Verhagen, J. (1987) Prostaglandins 34, 205–214.
[6] Kroegel, C., Yukawa, T., Dent, G., Chung, K.F. and Barnes, P.J. (1988) Immunol. 64, 559–562.
[7] Pincus, S.H. (1978) Blood 52, 127–134.
[8] Fukuda, T., Dunette, S.L., Reed, C.E., Ackerman, S.J., Peters, M.S. and Gleich, G.J. (1985) Am. Rev. Respir. Dis. 132, 981–988.
[9] Ward, S.G. and Westwick, J. (1988) Br. J. Pharmacol. 93, 769–774.
[10] Pollock, W.K., Rink, T.J. and Irvine, R.F. (1986) Biochem. J. 235, 869–877.
[11] Furchgott, R.F. (1972) in: Catecholamines (Blasko, H. and Muscholl, E. eds) pp.283–335, Springer, New York.
[12] Hille, B. (1984) in: Ionic Channels of Excitable Membranes, pp.273–279, Sinauer Associates Inc., Massachusetts.
[13] Casals-Stenzel, J., Muacevic, G. and Weber, K.H. (1987) J. Pharmacol. Exp. Ther. 241, 974–981.
[14] Ukena, D., Dent, G., Birke, B.W., Robaut, C., Sybrecht, G.W. and Barnes, P.J. (1988) FEBS Lett. 228, 285–289.
[15] Hallam, T.J., Sanchez, A. and Rink, T.J. (1984) Biochem. J. 218, 819–827.
[16] Ward, S.G., Please, R.D. and Westwick, J. (1988) Br. J. Pharmacol. 93, 74P.
[17] Conrad, G.W. and Rink, T.J. (1986) J. Cell. Biol. 103, 439–450.
[18] Kimani, G., Tonnesen, M.G. and Henson, P.M. (1988) J. Immunol. 140, 3161–3166.
[19] Tou, J.S. (1985) Biochem. Biophys. Res. Commun. 127, 1045–1051.
[20] Sage, S.O. and Rink, T.J. (1985) FEBS Lett. 188, 135–140.
[21] Hallam, T.J. and Rink, T.J. (1985) FEBS Lett. 186, 157–179.
[22] Poll, C. and Westwick, J. (1986) Br. J. Pharmacol. 88, 246P.