Biochem. J. (1985) **228**, 697–706
Printed in Great Britain

Effects of platelet-activating factor on the release of arachidonic acid and prostaglandins by rabbit iris smooth muscle

Inhibition by calcium channel antagonists

Sardar Y. K. YOUSUFZAI and Ata A. ABDEL-LATIF
Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30912, U.S.A.

(*Received 31 December 1984/11 February 1985; accepted 20 February 1985*)

Addition of physiological concentrations (10^{-12}–10^{-8} M) of platelet-activating factor (PAF) to rabbit iris muscle induced a rapid release (in 15s) of prostaglandin (PG)E_2 and 6-oxo-$PGF_{1\alpha}$, measured by radioimmunoassay and rapid release of ^{14}C-labelled arachidonate and PGE_2 in muscle prelabelled with [^{14}C]arachidonic acid, measured by radiochromatography. These PAF actions are concentration- and time-dependent. The effect of PAF on PG release is not mediated through the cyclo-oxygenase pathway. The studies on the properties and mechanism of arachidonate release from phosphatidylinositol and other phospholipids in prelabelled irides by PAF suggest the involvement of a phospholipase A_2. This conclusion is supported by the findings: (*a*) that both the removal of arachidonate and formation of lysophosphatidylinositol, from phosphatidylinositol, by PAF occur concomitantly in a time-dependent manner, (*b*) that Ca^{2+} is required for the agonist-induced release of arachidonate and PGE_2, and (*c*) that in contrast to the rapid release of [^{3}H]*myo*-inositol phosphates by carbachol and other Ca^{2+}-mobilizing agonists previously reported in the iris muscle [Akhtar & Abdel-Latif (1984) *Biochem. J.* **224**, 291–300], PAF (10^{-12}–10^{-8} M) did not appreciably enhance the release of [^{14}C]*myo*-inositol phosphates and ^{32}P labelling of phosphatidate and phosphatidylinositol in this tissue. Ca^{2+}-channel antagonists, such as nifedipine, verapamil, diltiazem and manganese inhibited PAF-induced arachidonate and PGE_2 release in a dose-dependent manner. K^+ depolarization, which causes influx of extracellular Ca^{2+} in smooth muscle, did not increase the release of arachidonate and PGE_2. The ability of Ca^{2+} antagonists to inhibit arachidonate release by PAF in this tissue probably reflects interference with PAF binding to its receptor. The PAF-induced release of arachidonate and PGE_2 occur independently of the cyclo-oxygenase and lipoxygenase pathways. Whether the PAF-induced release of arachidonate and PG in the iris muscle is involved in the pathogenesis of inflammatory and/or physiological reactions in the eye, and how much the inhibitory effects of Ca^{2+}-entry blockers on the PAF actions contribute to the therapeutic use of these drugs, remain to be established.

The platelet-activating factor (PAF) has been reported to possess inflammatory and hypotensive properties and to provoke cellular responses in a wide variety of tissues (for reviews see Vargaftig *et al.*, 1981; Pinckard *et al.*, 1982). It induces contraction in guinea pig ileal smooth muscle (Findlay *et al.*, 1981), promotes the degradation of phosphoinositides in platelets (Shukla & Hanahan, 1982; Ieyasu *et al.*, 1982; Mauco *et al.*, 1983; Billah & Lapetina, 1983; MacIntyre & Pollock, 1983) and stimulates the release of arachidonic acid in platelets (Shaw *et al.*, 1981). Furthermore, specific receptor sites for this bioactive lipid on platelet and smooth muscle membranes have been demonstrated (Hwang *et al.*, 1983). In platelets there is experimental evidence which suggests that PAF could exert its biological effects by increasing the permeability of membranes to Ca^{2+} (Lee *et al.*,

Abbreviations used: PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); PG, prostaglandin; RIA, radioimmunoassay.

1981). It is also possible that the increase in intracellular levels of Ca^{2+} that occurs during PAF-induced platelet aggregation is caused by an indirect effect of the lipid (Lee *et al.*, 1983; White, 1984; Shukla & Hanahan, 1984). However, while there is a considerable amount of information about the effects of PAF on several metabolic pathways in a variety of tissues, the mechanisms of action of this bioactive phospholipid are largely uninvestigated.

In the past several years, we have extensively studied the link between the activation of Ca^{2+}-mobilizing receptors, such as muscarinic cholinergic and α_1-adrenergic and the phosphodiesteratic cleavage of phosphoinositides (Abdel-Latif *et al.*, 1985; Akhtar & Abdel-Latif, 1984) and release of arachidonic acid for PG synthesis in the rabbit iris (Abdel-Latif & Smith, 1982; Abdel-Latif *et al.*, 1983; Yousufzai & Abdel-Latif, 1984). At present, there is limited information on the effects and mechanism of action of PAF on arachidonic acid release and PG synthesis in smooth muscle.

In the present study, we have investigated the properties and the mechanism of action of PAF on arachidonic acid release and PG synthesis in the rabbit iris. The results described in this paper indicate (*a*) that PAF stimulates the release of arachidonate and consequently PG synthesis in a time- and dose-dependent manner; (*b*) that arachidonate release by PAF is probably mediated through phospholipase A_2; and (*c*) that PAF-induced release of arachidonate is Ca^{2+}-dependent, is inhibited by Ca^{2+}-antagonistic drugs, and that it is not mediated through metabolites generated by the cyclo-oxygenase and lipoxygenase pathways.

Materials and methods

Chemicals

[1-^{14}C]Arachidonic acid (specific radioactivity 56.5mCi/mmol), [^{3}H]arachidonic acid (specific radioactivity 80–135Ci/mmol) and [U-^{14}C]*myo*-inositol (specific radioactivity 333mCi/mmol) were purchased from Amersham Corp., Arlington Heights, IL, U.S.A. Indomethacin, nordihydroguaiaretic acid and noradrenaline were purchased from Sigma Chemical Co. PGE_2 and 6-oxo-$PGF_{1\alpha}$ ^{3}H RIA kits were obtained from Seragen Inc., Boston, MA, U.S.A. PAF was obtained from Calbiochem, San Diego, CA, U.S.A. Lysophosphatidylinositol was purchased from Serdary Research Laboratories, London, Ontario, Canada. Nifedipine, verapamil and diltiazem were gifts from Pfizer, New York, NY, U.S.A., Knoll Pharmaceuticals Co., Whippany, NJ, U.S.A. and Marion Laboratories, Kansas City, MO, U.S.A. respectively. All other chemicals were of reagent grade.

Preparation of irides

Albino rabbits of either sex weighing approx. 2kg were used in the present work. The unanaesthetized rabbits were killed by a blow to the head followed by decapitation. The eyes were enucleated immediately and irides were removed and placed in pairs from the same animal in tubes containing 2ml of Krebs–Ringer bicarbonate buffer that contained 10mM-D-glucose. The pH of the Krebs–Ringer was adjusted to 7.4 with O_2/CO_2 (97:3).

Incubation and assay of release of PGE_2 and 6-oxo-$PGF_{1\alpha}$ by radioimmunoassay

In general, irides were incubated (one of the pair was used as control and the other as experimental) in 1ml of Krebs–Ringer bicarbonate buffer at 37°C for 10min or as indicated. PAF (bound to 25μg of bovine serum albumin) and other pharmacological agents were added as indicated. When PAF was employed, an equivalent amount of bovine serum albumin was routinely added to the control. The reactions were ended by acidification of the medium with 10% formic acid to pH3.5 and addition of ethyl acetate. The solvent was evaporated under N_2. The residue was dissolved in chloroform/methanol (2:1, v/v) and PG was determined by RIA as previously described (Yousufzai & Abdel-Latif, 1984). The amount of PG in each sample was determined by interpolation from the standard curve. Rate of PG release is presented as the amount of PG/10min per g of tissue or as indicated. Data presented in text and Figures are means ± S.E.M.

Preparation of microsomes and assay for the cyclo-oxygense

Microsomes were prepared from iris muscle as previously described (Abdel-Latif & Smith, 1982). Microsomes, equivalent to 0.6mg of protein, were incubated in 1ml of 0.1M-phosphate buffer, pH7.8, containing 1μM-arachidonic acid, in the presence and absence of various concentrations of PAF (10^{-12}–10^{-6}M) at 37°C for 10min. At the end of incubation the prostaglandins were extracted and analysed by RIA.

Prelabelling of iris with [^{14}C]arachidonic acid and assay for the release of labelled arachidonate and PGE_2

In general, two irides from the same rabbit were preincubated in 2ml of Krebs–Ringer bicarbonate buffer that contained 0.2μCi of [^{14}C]arachidonic acid/ml for 1h. The prelabelled irides were washed three times with non-radioactive medium, then

incubated (of the pair one was used as control) in the absence and presence of the drug as indicated. At the end of incubation the medium was analysed for radioactive arachidonic acid and PGE_2 and the tissue was analysed for phospholipids. The medium was acidified with 10% formic acid to pH 3.5 and extracted three times with 3ml of ethyl acetate. The solvent was evaporated under N_2. The residue was dissolved in chloroform/methanol (2:1, v/v), spotted on Whatman precoated silica gel LK6DF plates and developed in a solvent system (Hamberg & Sammuelsson, 1966) of ethyl acetate/acetic acid/trimethylpentane/water (11:2:5:10, by vol.). After visualization of the prostaglandin standards by exposure to I_2 vapour, the radioactive prostaglandin spots were located by autoradiography and the radioactive contents of PGE_2 and arachidonic acid were measured by counting in a Beckman liquid-scintillation counter.

Phospholipids were extracted twice from the tissue with chloroform/methanol/HCl (400:200:1.5, by vol.) and phospholipids were separated by two-dimensional t.l.c. as previously described (Abdel-Latif *et al.*, 1977).

Lysophosphatidylinositol was separated by one-dimensional t.l.c. on silica gel 60 high-performance thin-layer plates as previously described (Yousufzai & Abdel-Latif, 1984). Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Changes in the release of PGE_2 and 6-oxo-$PGF_{1\alpha}$ into the medium are presented as the amount of PG in μg/g of tissue, and changes in the release of arachidonate from prelabelled tissue into the medium are expressed as ^{14}C radioactivity (d.p.m.)/iris. The data presented in the Figures are representative of three or more similar experiments, and error bars represent the S.E.M. values calculated from at least three separate experiments. Statistical analysis of the data was done by Student's *t* test for paired differences.

Results

Dose–response to PAF for PG release

Previously, we have reported that rabbit irides spontaneously released PGE_2 and 6-oxo-$PGF_{1\alpha}$ into the medium, without addition of exogenous precursor, and that this increased with time of incubation up to 15min, then levelled off between 30 and 60min (Yousufzai & Abdel-Latif, 1984). As can be seen from Fig. 1, the release of PGE_2 was three to four times as high as that of 6-oxo-$PGF_{1\alpha}$. The release of both PGE_2 and 6-oxo-$PGF_{1\alpha}$ was significantly enhanced when PAF was added to the incubation medium, and the PAF-induced production of these PGs by the iris increased in a dose-dependent manner (Fig. 1).

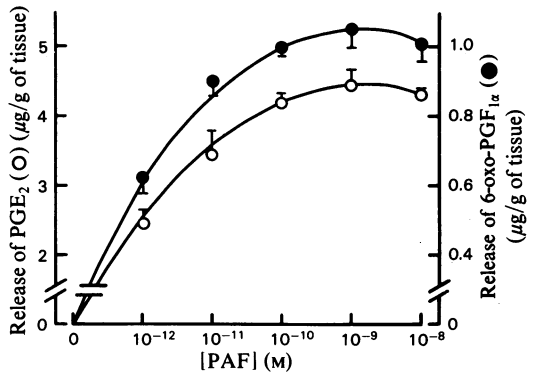

Fig. 1. *Dose–response to PAF for release of PGE_2 and 6-oxo-$PGF_{1\alpha}$ in the iris muscle*

Irides (of the pair, one was used as control and the other as experimental) were incubated in 1ml of Krebs–Ringer bicarbonate buffer (pH 7.4) in the absence and presence of various concentrations of PAF as indicated for 10min at 37°C. At the end of incubation PG in the medium was quantified by RIA. The amounts of PG released are expressed as μg/g of tissue. In general, after 10min of incubation, the basal release of PGE_2 and 6-oxo-$PGF_{1\alpha}$ (μg/g of tissue) were 2.5 ± 0.1 and 0.65 ± 0.05, respectively). Each point is the mean of values from four separate experiments conducted in triplicate.

Maximal stimulation of PG production was observed at PAF concentrations between 10^{-10} and 10^{-8}M. At 10^{-9}M, PAF increased the release of PGE_2 and 6-oxo-$PGF_{1\alpha}$ by 67 and 51%, respectively. Since the effect of PAF on PGE_2 release was more pronounced than that on 6-oxo-$PGF_{1\alpha}$, in the following studies we have routinely assayed for PGE_2. These data indicate that the PAF-stimulated release of PG in the iris is concentration-dependent.

Time-course for the effect of PAF on the release of PGE_2 and 6-oxo-$PGF_{1\alpha}$

The time course for the action of 10^{-9}M-PAF on the release of PGE_2 and 6-oxo-$PGF_{1\alpha}$ is shown in Fig. 2. A detectable increase in the release of PGE_2 (11%) and 6-oxo-$PGF_{1\alpha}$ (8%) was observed within 15s after addition of PAF and maximal increase in the agonist-stimulated release of PG (56–67%) was reached between 5 and 10min of incubation. These data indicate that the PAF-stimulated release of PG in the iris is time-dependent.

Dose–response to PAF for release of ^{14}C-labelled arachidonic acid and PGE_2

There is general agreement now that PG formation must be preceded by a lipolytic process to release free arachidonic acid from the tissue phospholipids (for review see Irvine, 1982). To

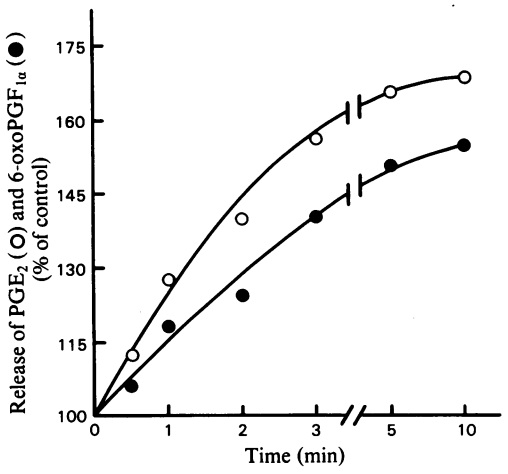

Fig. 2. *Time course for the effect of PAF on the release of PGE_2 and 6-oxo-$PGF_{1\alpha}$ in the iris muscle*
Conditions of incubation were the same as described under Fig. 1 except that the experimental contained 10^{-9}M-PAF and the incubations were carried out for various time intervals as indicated. Each point is the mean of values from two separate experiments conducted in triplicate.

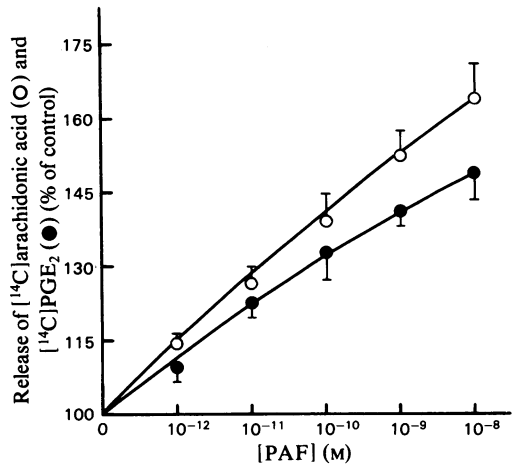

Fig. 3. *Dose–response to PAF for release of ^{14}C-labelled arachidonic acid and PGE_2 in iris muscle*
Irides (in pairs) were preincubated for 1 h in 2 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.2 μCi of [^{14}C]arachidonic acid/ml. The prelabelled irides were washed twice with non-radioactive buffer, then incubated in the absence and presence of various concentrations of PAF as indicated for 10 min at 37°C. Extraction of arachidonic acid and PG from the medium and analysis of radioactivity were as described in the Materials and methods section. Typical control values (in absence of PAF) for radioactive arachidonic acid and PGE_2 were (d.p.m./iris) 13257 ± 985 and 2298 ± 137, respectively. The effects of PAF on arachidonic acid and PGE_2 release are expressed as percentages of their respective controls. Each point is the mean of values from three separate experiments run in triplicate.

investigate the mechanism of action of PAF on PG synthesis, irides were prelabelled with [^{14}C]arachidonic acid and the effects of various concentrations of PAF on the release of radioactivity was determined by radiochromatography. As can be seen from Fig. 3, PAF increased the release of labelled arachidonate and PGE_2 in a dose-dependent manner. Thus, PAF at 10^{-12} and 10^{-9}M increased arachidonate release by 15 and 53%, respectively. These data indicate that release of labelled arachidonate and PGE_2 from prelabelled membrane phospholipids by PAF is dose-dependent.

Time course for the effect of PAF on the release of ^{14}C-labelled arachidonic acid and PGE_2

The time course for the action of 10^{-9}M-PAF on the release of labelled arachidonate and PGE_2 from irides prelabelled with [^{14}C]arachidonic acid is given in Fig. 4. Significant increase in the release of labelled arachidonate and PGE_2 was observed within 1 min after addition of 10^{-9}M-PAF and after 10 min this increased to 64 and 71% of their respective controls, respectively. These data indicate that the release of radioactive arachidonate from prelabelled membrane phospholipids by PAF is time-dependent.

Effects of PAF on conversion of arachidonic acid into PGE_2 by iris microsomes

The increase in the release of PGE_2 in the iris muscle by PAF (Figs. 1–4) could occur in two ways: (*a*) the bioactive lipid could act to stimulate the cyclo-oxygenase pathway; and (*b*) it could stimulate the phospholipase pathways to release arachidonate for PG synthesis. To answer the first possibility we have investigated the effects of various concentrations of PAF (10^{-12}–10^{-6}M) on the conversion of arachidonate into PGE_2 by iris microsomes. We found no effect of PAF on the conversion of arachidonate into PGE_2 by iris microsomes at all concentrations investigated. Thus, PGE_2 synthesis by iris microsomes, measured by RIA, in the absence and presence of 10^{-9}M-PAF was found to be (ng/mg of protein): 8.13 ± 0.123 and 7.98 ± 0.42, respectively.

Time course for the effect of PAF on the breakdown of doubly labelled phosphatidylinositol and release of arachidonate in the iris muscle

To answer the second possibility raised above, namely the PAF could act by stimulating the phospholipase pathway to increase the release of free arachidonate and consequently PG synthesis,

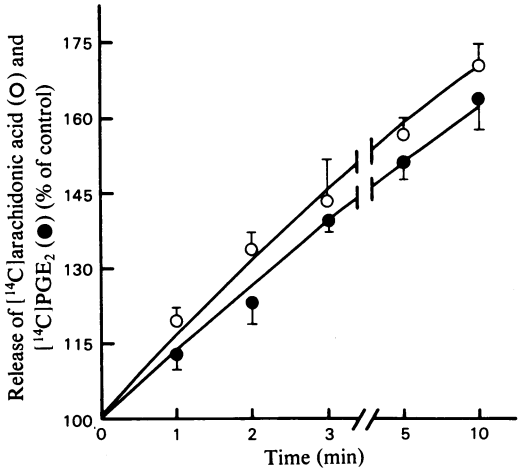

Fig. 4. *Time course for the effect of PAF on the release of ^{14}C-labelled arachidonic acid and PGE_2 in iris muscle*
Conditions of incubation were the same as described under Fig. 3 except that the experimental contained 10^{-9}M-PAF and the incubations were carried out for various time intervals as indicated. The effects of PAF on arachidonate and PGE_2 release are expressed as percentages of their respective controls. Each point is the mean of values from three separate experiments run in triplicate.

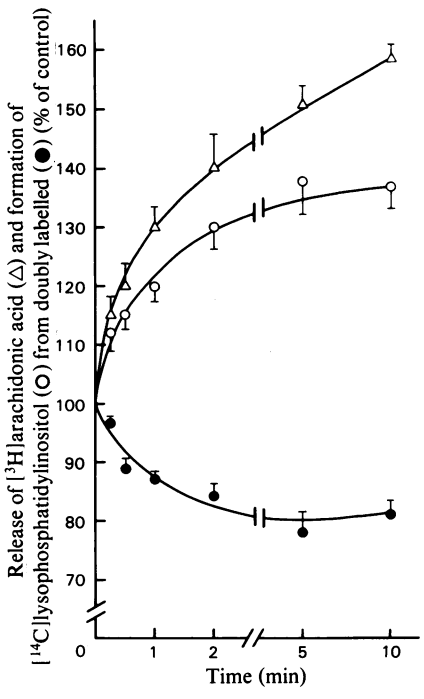

Fig. 5. *Time course for the effect of PAF on the release of ^{3}H-labelled arachidonate and formation of ^{14}C-labelled lysophosphatidylinositol from doubly labelled phosphatidylinositol*
Irides (in pairs) were preincubated for 2h in 2ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing 2μCi of [^{3}H]arachidonic acid and 0.3μCi of [^{14}C]*myo*-inositol. The doubly prelabelled irides were washed twice with non-radioactive buffer, then incubated in the absence and presence of PAF (10^{-9}M) for various time intervals as indicated. Extractions of [^{3}H]arachidonic acid from the medium and phospholipids from the tissue and analysis of radioactivity were as described in the Materials and methods section. The control values (zero time) for ^{14}C radioactivities in lysophosphatidylinositol and in doubly labelled phosphatidylinositol were (d.p.m./iris) 5451 ± 165 and 20395 ± 726, respectively. The effects of PAF on arachidonic acid release and on the formation of lysophosphatidylinositol from phosphatidylinositol are expressed as percentages of their respective controls. Each point is the mean of values from two separate experiments run in triplicate.

we have investigated the time course for the effect of PAF on the breakdown of phosphatidylinositol in iris muscle prelabelled with [^{3}H]arachidonic acid and [^{14}C]*myo*-inositol. Within 15s PAF (10^{-9}M) increased the release of [^{3}H]arachidonate and formation of [^{14}C]lysophosphatidylinositol from doubly labelled phosphatidylinositol by 15% and 10%, respectively and after 10min of incubation the increases were 58% and 38%, respectively (Fig. 5). Lysophosphatidylinositol could be degraded further into monoacylglycerol and inositol phosphate (Murase & Okuyama, 1985). Under the same experimental conditions, we were able to observe only slight changes in the release of [^{14}C]*myo*-inositol phosphates by PAF (results not shown). Analysis of ^{14}C radioactivity in phosphatidylcholine and phosphatidylethanolamine in iris muscle prelabelled with [^{14}C]arachidonic acid revealed a loss of about 3% of radioactivity from these phospholipids upon incubation for 1–10min with PAF (results not shown). These data suggest that PAF acts to release arachidonate from phosphatidylinositol, and probably other phospholipids, by stimulating the phospholipase A_2 pathway.

Effect of PAF, in the absence and presence of Ca^{2+}, on release of [^{14}C]arachidonate and PGE_2

In light of our previous studies with neurotransmitters and PG synthesis in the iris (Yousufzai & Abdel-Latif, 1984) and the present findings with PAF (Fig. 5), both of which provided evidence that a phospholipase A_2 hydrolysing phosphatidylinositol is activated when the iris is stimulated for PG synthesis, we decided to investigate the role of Ca^{2+} in the PAF-induced release of arachidonate and PG in this tissue. As can be seen from Table 1, in the absence of Ca^{2+} PAF had no effect on the release of PGE_2 and [^{14}C]arachidonic acid. How-

Table 1. *Effects of PAF, in the absence and presence of Ca^{2+}, on (a) the release of PGE_2 and (b) the release of ^{14}C-labelled arachidonate from rabbit iris*

(*a*) For the studies on the effects of Ca^{2+} and PAF on PGE_2 release, irides (in pairs) were first washed with Ca^{2+}-free Krebs–Ringer bicarbonate buffer containing EGTA (0.25 mM), then with buffer alone to remove the chelator. One iris from each of the washed pairs was incubated in the Ca^{2+}-free buffer in the absence and presence of PAF and/or Ca^{2+} as indicated for 10 min at 37°C. PGE_2 in the medium was quantified by RIA. (*b*) For the studies on the effects of Ca^{2+} and PAF on arachidonic acid release, irides (in pairs) were preincubated for 1 h in 2 ml of Ca^{2+}-free Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.2 μCi of [^{14}C]arachidonic acid/ml. The prelabelled irides were washed twice with Ca^{2+}-free buffer containing EGTA (0.25 mM), then with Ca^{2+}-free buffer to remove the EGTA. One iris from each of the washed pairs was incubated in the Ca^{2+}-free buffer in the absence and presence of PAF and/or Ca^{2+} as indicated for 10 min at 37°C. Extraction of arachidonic acid from the medium and analysis of radioactivity were as described in the Materials and methods section. The effects of PAF and Ca^{2+} on the release of PGE_2 and [^{14}C]arachidonic acid are expressed as percentages of their respective controls. Each value is the mean of values from two separate experiments run in triplicate. Significance of difference between paired data was calculated by using Student's *t* test; $*P<0.01$, $**P<0.02$.

Additions	(*a*) PGE_2 release		(*b*) [^{14}C]Arachidonic acid release	
	(μg/g of tissue)	(% of control)	(d.p.m./iris)	(% of control)
Control (Ca^{2+}-free medium)	2.01 ± 0.12	100	5134 ± 204	100
PAF (10^{-9} M)	2.15 ± 0.11	107	5545 ± 173	108
Ca^{2+} (1.25 mM)	2.75 ± 0.14**	137	6674 ± 221*	130
PAF (10^{-9} M) + Ca^{2+} (1.25 mM)	4.13 ± 0.18*	205	9482 ± 311*	185

ever, when Ca^{2+} was added to the incubation medium the release of PGE_2 and [^{14}C]arachidonate by the agonist increased by 105 and 85%, respectively. These data indicate that Ca^{2+} is required for the PAF-induced arachidonate release in this tissue.

Effect of concentration of nifedipine on the PAF-induced release of ^{14}C-labelled arachidonic acid and PGE_2

In view of the finding that Ca^{2+} is required for PAF-induced release of arachidonate and PGE_2 (Table 1), it was of interest to determine the effects of nifedipine, a Ca^{2+}-channel antagonist, on their release in the iris. As can be seen from Fig. 6, nifedipine inhibited PAF-induced release of [^{14}C]arachidonic acid and [^{14}C]PGE_2 in a dose-dependent manner. Thus, at 10^{-9} M-nifedipine the inhibition of arachidonate and PGE_2 release was 12 and 33%, respectively and at 10^{-7} M it was 33 and 55%, respectively. Nifedipine alone had no effect on the basal release of ^{14}C radioactivity (see Table 2 below). Under the same experimental conditions nifedipine (1 μM) had no effect on acetylcholine (50 μM) and noradrenaline (50 μM)-induced release of labelled arachidonate (S. Y. K. Yousufzai & A. A. Abdel-Latif, unpublished work).

Effects of various Ca^{2+}-channel antagonists on PAF-induced PGE_2 release

The effects of various Ca^{2+}-channel antagonists on PAF-induced release of PGE_2 are given in Table 2. Neither nifedipine alone, nor verapamil alone showed any effect on PAF-induced release of

Table 2. *Effects of various Ca^{2+}-channel antagonists on PAF-induced release of PGE_2 from iris muscle*

Irides (of the pair, one was used as control and the other as experimental) were incubated in 1 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) in the absence and presence of various concentrations of Ca^{2+}-channel antagonists as indicated for 10 min at 37°C. At the end of incubation PG in the medium was quantified by RIA. The amounts of PG released are expressed as μg/g of tissue. Each value is the mean of values from two separate experiments conducted in triplicate. Significance of difference between paired data was calculated by using Student's *t* test: $*P<0.01$, $**P<0.05$.

Additions	PGE_2 (μg/g of tissue)	Effects of blockers (% of control)
Control	2.43 ± 0.08	100
PAF (10^{-9} M)	4.12 ± 0.10*	170
Nifedipine (1 μM)	2.45 ± 0.10	100
PAF + nifedipine (0.1 μM)	2.93 ± 0.11**	121
PAF + nifedipine (1 μM)	2.27 ± 0.14	93
Verapamil (5 μM)	2.39 ± 0.13	98
PAF + verapamil (5 μM)	2.99 ± 0.10**	123
PAF + verapamil (25 μM)	2.37 ± 0.15	98
PAF + diltiazem (1 μM)	3.04 ± 0.02*	125
PAF + diltiazem (5 μM)	2.47 ± 0.09	102
PAF + manganese (0.25 mM)	2.88 ± 0.13**	119
PAF + manganese (0.5 mM)	2.52 ± 0.07	104

PGE_2; the effects of the other blockers alone were not investigated. The stimulatory effect of PAF (10^{-9} M) on PGE_2 release was completely blocked by 1 μM-nifedipine, 25 μM-verapamil, 5 μM-diltia-

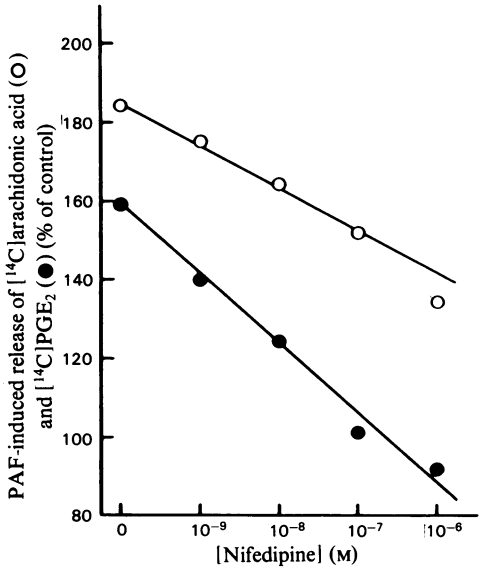

Fig. 6. *Dose–response curves for inhibition of PAF-induced release of ^{14}C-labelled arachidonic acid and PGE_2 by nifedipine in iris muscle*

Irides (in pairs) were preincubated for 1 h in 2 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.2 μCi of [^{14}C]arachidonic acid/ml. The prelabelled irides were washed twice with non-radioactive buffer, then incubated (in the dark) in the absence and presence of PAF (10^{-9} M) and various concentrations of nifedipine for 10 min at 37°C. Extraction of arachidonic acid and PG from the medium and analysis of radioactivity were as described in the Materials and methods section. The inhibitory effects of nifedipine on PAF-induced release of arachidonic acid and PGE_2 are expressed as percentages of their respective controls. Each point is the mean of values from two separate experiments run in triplicate.

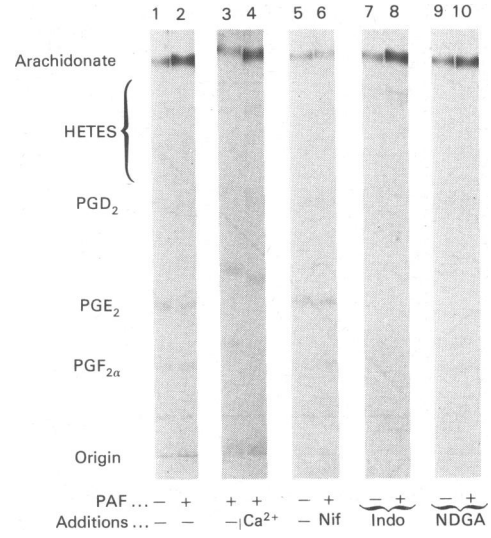

Fig. 7. *Typical autoradiographs after lipid extraction from the medium and TLC showing the effects of Ca^{2+}, nifedipine (Nif), indomethacin (Indo) and nordihydroguaiaretic acid (NDGA) on PAF-stimulated release of [^{14}C]arachidonic acid in iris muscle*

Autoradiographs showing the effects of 10^{-9} M-PAF (lanes 1 and 2) and PAF + 1.25 mM-Ca^{2+} (lanes 3 and 4) were taken from experiments similar to those described in Table 1, and the autoradiographs showing the effects of 1 μM-nifedipine (lanes 5 and 6) were taken from experiments similar to those described in Fig. 6. Conditions of incubation for the studies on the effects of 1.6 μM-indomethacin (lanes 7 and 8) and 1 μM-NDGA (lanes 9 and 10) were the same as described under Fig. 3 except that incubations were carried out in a medium containing indomethacin or NDGA as indicated. Abbreviation used: HETES, 5- and 12-hydroxy-6,8,11,14-eicosatetraenoic acids (5- and 12-HETE).

zem and 0.5 mM-manganese (Table 2). These studies indicate that, in the iris muscle, PAF actions on the release of arachidonate and PG are inhibited by Ca^{2+}-channel antagonists (Fig. 6 and Table 2).

Effects of indomethacin and nordihydroguaiaretic acid on PAF-stimulated release of arachidonic acid

It has been suggested that metabolites generated from arachidonic acid through the cyclo-oxygenase pathway (Mendlovic *et al.*, 1984) and through the lipoxygenase pathway (Lee *et al.*, 1983) could be involved in the actions of PAF in liver and platelets, respectively. In Fig. 7 we show the effects of Ca^{2+}, nifedipine (both of which were discussed above in Tables 1 and 2, and Fig. 6), indomethacin (a cyclo-oxygenase inhibitor) and nordihydroguaiaretic acid (a lipoxygenase inhibitor), on PAF-stimulated release of labelled arachidonate and its metabolites. Neither indomethacin nor nordihydroguaiaretic acid had any inhibitory effect on PAF actions. The increase in the radioactivity of arachidonic acid release by PAF in the presence of these inhibitors is due to their known inhibitory effects on the cyclo-oxygenase and lipoxygenase pathways. Thus, the effects of various concentrations of PAF on [^{14}C]arachidonic acid release in indomethacin-treated iris (Fig. 8) were significantly higher than those which were obtained in the absence of the inhibitor (Fig. 3). These data indicate that the metabolites of arachidonic acid are not involved in the PAF-induced release of arachidonic acid and PGE_2 in the iris muscle.

Discussion

In the present study, we have demonstrated that in the iris muscle physiological concentrations of PAF (10^{-12}–10^{-8} M) induced a rapid release (in

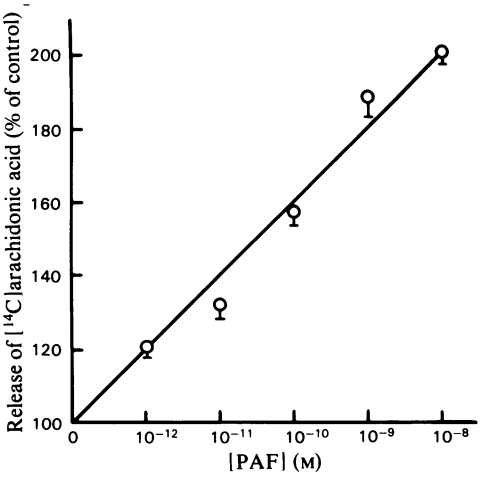

Fig. 8. *Dose–response to PAF for release of ^{14}C-labelled arachidonic acid in iris muscle treated with indomethacin*
Conditions of incubation were the same as described under Fig. 3 except that incubations were carried out in a medium containing 1.6 μM-indomethacin. Control value (after 10 min) for [^{14}C]arachidonic acid release was (d.p.m./iris) 16165 ± 1133. Each point is the mean of values from two separate experiments run in duplicate.

15 s) of PGE_2 and 6-oxo-$PGF_{1\alpha}$, measured by RIA, and rapid release of ^{14}C-labelled arachidonate and PGE_2, measured by radiochromatography. These PAF actions are concentration- and time dependent (Figs. 1–4). The effect of PAF on PG release is not mediated through the cyclo-oxygenase pathway, since the bioactive phospholipid had no effect on the conversion of arachidonate into PGE_2 by iris microsomes. These observations indicate that PAF acts on the release of arachidonate from membrane phospholipids, rather than on the metabolism of this polyunsaturated fatty acid. PAF has been reported to activate the release of arachidonate and its metabolites in rabbit platelets (Shaw *et al.*, 1981), in rabbit neutrophils (Chilton *et al.*, 1982) and in Swiss mouse 3T3 fibroblasts (Kawaguchi & Hisakawa, 1984).

The release of arachidonate from phosphoinositides could occur either through a direct release by phospholipase A_2 (Hong & Deykin, 1981; Billah & Lapetina, 1982), or through an indirect release by phosphatidylinositol-specific phospholipase C which produces diacylglycerol, followed by the actions of diacylglycerol and monoacylglycerol lipases to release arachidonate (Okazaki *et al.*, 1981; Prescott & Majerus, 1983). The data presented here on the mechanism of arachidonate release by PAF suggest the involvement of a phospholipase A_2. This conclusion is based on the following observations in the present work. (*a*) Both the removal of arachidonate and the formation of lysophosphatidylinositol from phosphatidylinositol by PAF occurred concomitantly in a time-dependent manner (Fig. 5). The finding that PAF increased the release of arachidonate from phosphatidylinositol by 58% (Fig. 5), compared with about 3% from phosphatidylcholine and phosphatidylethanolamine, suggests that phosphoinositides could serve as a major source for arachidonate in PG synthesis in this tissue. Efforts are now being made to determine the role of polyphosphoinositides in arachidonate release in the iris. Formation of lysophosphatidylinositol in transformed BALB/3T3 cells (Hong & Deykin, 1981) and platelets (Billah & Lapetina, 1982) is stimulated by thrombin and ionophore A23187. In rat gastric mucosa homogenates, degradation of phosphatidylinositol by phospholipase A_2 was 8–10-fold faster than that by phospholipase C (Wassef & Horwitz, 1981). The ratio between these lipases has not yet been determined in the iris muscle. However, in this muscle phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine are enriched in arachidonate (Yousufzai & Abdel-Latif, 1984). Failure to observe more pronounced deacylation of phosphatidylcholine and phosphatidylethanolamine by phospholipase A_2 in a variety of tissues, including the iris, is probably due to the rapid reacylation of their respective lysophospholipids (Walsh *et al.*, 1983; Chilton *et al.*, 1984). (*b*) Another observation which is in accord with the hypothesis that phospholipase A_2 is probably involved in PAF actions in this tissue is the finding that Ca^{2+} is required for the release of arachidonate by this agonist (Table 1). A major role proposed for Ca^{2+} is activation of phospholipase A_2 and specific release of arachidonic acid (Van den Bosch, 1980). PAF has been reported to promote Ca^{2+} uptake into platelets (Lee *et al.*, 1981, 1983; Hallam *et al.*, 1984). Rittenhouse-Simmons & Deykin (1978) proposed a simple model in which the activity of platelet phospholipase A_2 was thought to be regulated by free Ca^{2+} in the cytosol. However, while most phospholipases are Ca^{2+}-dependent, it does not necessarily mean that they are Ca^{2+}-controlled (Irvine, 1982). Thus, further studies are needed before a precise role for Ca^{2+} in PAF actions in the iris muscle can be established.

The finding that Ca^{2+}-channel antagonists, such as nifedipine, verapamil, diltiazem and manganese, inhibited PAF-induced arachidonic acid and PGE_2 release (Figs. 6 and 7, Table 2) could represent another mode of action of these agents in smooth muscle. In the iris, inhibition of PAF actions by Ca^{2+} antagonists could occur either by blocking the cation entry into the cell, or by interference with PAF binding to its receptor.

Although suppression of the voltage-activated inward displacement of Ca^{2+} is undoubtedly the most widely discussed property of these antagonists in smooth muscle, some of these drugs exhibit other properties, for example nifedipine and verapamil bind α receptors and muscarinic receptors in brain, and diltiazem slows the release of Ca^{2+} from smooth muscle sarcoplasmic reticulum (Nayler, 1982; Janis & Triggle, 1983). K^+ depolarization, which causes influx of extracellular Ca^{2+} in smooth muscle, had no effect on the release of labelled arachidonate in iris muscle prelabelled with [^{14}C]arachidonate (S. Y. K. Yousufzai, S. Naderi & A. A. Abdel-Latif, unpublished work). This finding suggests that arachidonate release by PAF is probably not directly linked to the voltage-dependent Ca^{2+} channels in this tissue. Thus, the ability of Ca^{2+} antagonists to inhibit arachidonate release by PAF in the iris muscle probably reflects interference with PAF binding to its receptor rather than an action at the voltage-dependent Ca^{2+} channels. Hallam *et al.* (1984) also inferred that verapamil could inhibit platelet response to PAF by interference with PAF binding to its receptors.

Arachidonate release may also be promoted by action of lipases on the diacylglycerol formed as a result of phosphoinositide breakdown (see above). PAF has been reported to promote the phosphodiesteratic cleavage of phosphoinositides in platelets (Shukla & Hanahan, 1982; Lapetina, 1982; Mauco *et al.*, 1983). However, preliminary studies revealed only minor changes in ^{32}P labelling of phosphatidate and phosphoinositides and in accumulation of [^{32}H]*myo*-inositol phosphates by various concentrations (10^{-12}–5×10^{-5} M) of PAF in the iris muscle (S. Y. K. Yousufzai & A. A. Abdel-Latif, unpublished work). This is in contrast with the rapid accumulation of [^{3}H]*myo*-inositol phosphates and the enhanced ^{32}P labelling of phosphoinositides and phosphatidate by carbachol and other Ca^{2+}-mobilizing agonists in this tissue (Abdel-Latif *et al.*, 1977, 1985; Akhtar & Abdel-Latif, 1984). Furthermore, while PAF produced ileal muscle contraction (Findlay *et al.*, 1981), the bioactive phospholipid alone had no effect on muscle contraction in porcine carotid arteries (Kester *et al.*, 1984), or in the rabbit iris smooth muscle (R. A. Akhtar, R. Gracy & A. A. Abdel-Latif, unpublished work). These observations suggest that in the iris muscle PAF does not behave like a typical Ca^{2+}-mobilizing agonist, and thus the role of the phosphatidylinositol-specific phospholipase C pathway in PAF-induced arachidonate release is probably not a major one for this agonist in the iris. This is in contrast to acetylcholine- and noradrenaline-induced release of arachidonate in the iris muscle, where both pathways are clearly available to the neurotransmitters (Yousufzai & Abdel-Latif, 1984).

The findings that indomethacin, a cyclo-oxygenase inhibitor, and nordihydroguaiaretic acid, a lipoxygenase inhibitor, had a stimulatory, rather than inhibitory, effect on arachidonate release by PAF (Figs. 7 and 8) suggest that metabolites generated from arachidonate through these pathways are not involved in the agonist actions on the iris muscle. PAF-induced platelet aggregation and release reaction occur independently of the cyclo-oxygenase (Shaw *et al.*, 1978; Cazenave *et al.*, 1979) or lipoxygenase (Lapetina, 1982) pathways.

In conclusion, we have shown that in the rabbit iris muscle, PAF, a potential mediator of inflammation and allergic responses, promotes a rapid release of arachidonic acid and prostaglandins. Activation of PAF receptors provokes the release of arachidonate from membrane phosphatidylinositol and other phospholipids, probably via activation of phospholipase A_2, and subsequently this leads to the observed increase in PG release. Release of arachidonate and PG by PAF is Ca^{2+}-dependent, it is inhibited by Ca^{2+}-channel antagonists and it is not influenced by cyclo-oxygenase and lipoxygenase inhibitors. Whether the PAF-induced release of arachidonic acid and PG in the iris muscle is involved in the pathogenesis of inflammatory and/or physiological reactions in the eye, and how much the inhibitory effects of the Ca^{2+} entry blockers on the observed PAF actions contribute to the well-known therapeutic use of Ca^{2+}-antagonistic drugs, remains to be established.

This work was supported by USPHS grants EY-04387 and EY-04171 from the National Eye Institute. This is contribution no. 0882 from the Department of Cell and Molecular Biology, Medical College of Georgia. We are grateful to Ronald Gracy and Robert Reps for technical assistance.

References

Abdel-Latif, A. A. & Smith, J. P. (1982) *Biochim. Biophys. Acta* **711**, 478–489

Abdel-Latif, A. A., Akhtar, R. A. & Hawthorne, J. N. (1977) *Biochem. J.* **162**, 61–73

Abdel-Latif, A. A., Smith, J. P., Yousufzai, S. Y. K. & Dover, R. K. (1983) in *Neural Membranes* (Sun, G. Y., Bazan, N., Wu, J.-Y., Porcellati, G. & Sun, A. Y., eds.), pp. 97–122, The Humana Press, Clifton, NJ

Abdel-Latif, A. A. Smith, J. P. & Akhtar, R. A. (1985) in *The Chilton Conference on Inositol and Phosphoinositides* (Bleasdale, J. E., Eichberg, J. & Hauser, G., eds.), pp. 275–298, The Humana Press, Clifton, NJ

Akhtar, R. A. & Abdel-Latif, A. A. (1984) *Biochem. J.* **224**, 291–300

Billah, M. M. & Lapetina, E. G. (1982) *J. Biol. Chem.* **257**, 5169–5200

Billah, M. M. & Lapetina, E. G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 965–968
Cazenave, J. P., Benveniste, J. & Mustard, J. F. (1979) *Lab. Invest.* **41**, 275–285
Chilton, F. H., O'Flaherty, J. T., Walsh, C. E., Thomas, M. J., Wykle, R. L., DeChatelet, L. R. & Waite, M. (1982) *J. Biol. Chem.* **257**, 5402–5407
Chilton, F. H., Ellis, J. M., Olson, S. C. & Wykle, R. L. (1984) *J. Biol. Chem.* **259**, 12014–12019
Findlay, S. R., Lichtenstein, L. M., Hanahan, D. J. & Pickard, R. N. (1981) *Am. J. Physiol.* **241**, C130–C134
Hallam, T. J., Sanchez, A. & Rink, T. J. (1984) *Biochem. J.* **218**, 819–827
Hamberg, M. & Sammuelsson, B. (1966) *J. Biol. Chem.* **241**, 257–263
Hong, S. L. & Deykin, D. (1981) *J. Biol. Chem.* **256**, 5215–5219
Hwang, S.-B., Lee, C.-H., Cheah, M. J. & Shen, T. Y. (1983) *Biochemistry* **22**, 4756–4763
Ieyasu, H., Takai, Y., Kaibuchi, K., Sawamura, M. & Nishizuka, Y. (1982) *Biochem. Biophys. Res. Commun.* **108**, 1701–1708
Irvine, R. F. (1982) *Biochem. J.* **204**, 3–16
Janis, R. A. & Triggle, D. J. (1983) *J. Med. Chem.* **26**, 775–785
Kawaguchi, H. & Hisakawa, Y. (1984) *FEBS Lett.* **176**, 93–96
Kester, M., Ledvora, R. F. & Barany, M. (1984) *Eur. J. Physiol.* **400**, 209–202
Lapetina, E. G. (1982) *J. Biol. Chem.* **257**, 7314–7317
Lee, T.-C., Malone, B., Blank, M. L. & Snyder, F. (1981) *Biochem. Biophys. Res. Commun.* **102**, 1262–1268
Lee, T.-C., Malone, B. & Snyder, F. (1983) *Arch. Biochem. Biophys.* **223**, 33–39
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
MacIntyre, D. E. & Pollock, W. K. (1983) *Biochem. J.* **212**, 433–437
Mauco, G., Chap, H. & Douste-Blazy, L. (1983) *FEBS Lett.* **153**, 361–365
Mendlovic, F., Corvera, S. & Garcia-Sainz, J. A. (1984) *Biochem. Biophys. Res. Commun.* **123**, 507–514
Murase, S. & Okuyama, H. (1985) *J. Biol. Chem.* **260**, 262–265
Nayler, W. G. (1982) in *Calcium Regulation by Calcium Antagonists* (Rahwan, R. G. & Witiak, D. T., eds.), pp. 1–16, American Chemical Society Publishers, Washington, DC
Okazaki, T., Sagawa, N., Okita, J. R., Bleasdale, J. E., McDonald, P. C. & Johnston, J. M. (1981) *J. Biol. Chem.* **256**, 7316–7321
Pinckard, R. N., McManus, L. M. & Hanahan, D. J. (1982) *Adv. Inflam. Res.* **4**, 147–180
Prescott, S. M. & Majerus, P. W. (1983) *J. Biol. Chem.* **258**, 764–769
Rittenhouse-Simmons, S. & Deykin, D. (1978) *Biochim. Biophys. Acta* **543**, 409–422
Shaw, J. O., Printz, M. P., Hirabayashi, K. & Henson, P. M. (1978) *J. Immunol.* **121**, 1939–1945
Shaw, J. O., Klusick, S. J. & Hanahan, D. J. (1981) *Biochim. Biophys. Acta* **663**, 222–229
Shukla, S. D. & Hanahan, D. J. (1982) *Biochem. Biophys. Res. Commun.* **106**, 697–703
Shukla, S. D. & Hanahan, D. J. (1984) *Arch. Biochem. Biophys.* **232**, 458–466
Van den Bosch, H. (1980) *Biochim. Biophys. Acta* **604**, 191–246
Vargaftig, B. B., Chignard, M., Benveniste, J., Lefort, J. & Wal, F. (1981) *Ann. N.Y. Acad. Sci.* **370**, 119–137
Walsh, C. E., DeChatelet, L. R., Chilton, F. H., Wykle, R. L. & Waite, M. (1983) *Biochim. Biophys. Acta* **750**, 32–40
Wassef, M. K. & Horowitz, M. I. (1981) *Biochim. Biophys. Acta* **665**, 234–243
White, G. C. (1984) *Biochem. Biophys. Res. Commun.* **120**, 474–480
Yousufzai, S. Y. K. & Abdel-Latif, A. A. (1984) *Prostaglandins* **28**, 399–415
Yousufzai, S. Y. K. & Abdel-Latif, A. A. (1985) *Biochem. Pharmacol.* **34**, 539–544