1-*O*-alkyl-2-acetyl-*sn*-glycerol: a platelet-activating factor metabolite with biological activity in vascular smooth muscle cells

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Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF) is a potent vasoactive ether lipid produced by activated blood cells and endothelial cells. Vascular smooth muscle cells partially convert exogenous PAF to 1-*O*-alkyl-2-acetyl-*sn*-glycerol (AAG), a biologically active diacylglycerol analogue. AAG is formed rapidly (<15 s) after exposure of the smooth muscle cells and does not appear to be a substrate for diacylglycerol kinase in these cells. Although most of the compound is metabolized to 1-*O*-alkyl-*sn*-glycerol, a small quantity remains as AAG for ≥ 6 h. AAG inhibits phorbol ester binding, and it is as effective an activator of protein kinase C as diolein in an in vitro assay. Furthermore, AAG and PAF produce the same pattern of effects on smooth muscle cell proliferation. These observations suggest that at least some of the actions of PAF in vascular smooth muscle may be mediated through the formation of AAG, a stable, bioactive metabolite that appears to function as a diacylglycerol analogue.

Introduction

We have recently reported the molecular trafficking and metabolism of 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor; PAF)¹ by endothelial and vascular smooth muscle cells in a coculture model system designed to study cell-cell interactions in the vascular wall (Stoll and Spector, 1989). While characterizing the effects of PAF in this system, we noted that PAF produced a proliferative response in the smooth muscle cells and was partially converted to a me-

tabolite that appeared to be a diacylglycerol analogue. Diacylglycerols are important intracellular second messengers that activate protein kinase C (Nishizuka, 1984, 1986, 1988; Rando, 1988). If the PAF metabolite observed in vascular cells were in fact a diacylglycerol analogue, it might also be expected to have some second-messenger activity and possibly to mediate the proliferative response. This possibility was strengthened by the close structural similarity between 1-*O*-alkyl-2-acetyl-*sn*-glycerol, a likely diacylglycerol-like metabolite that could be derived from PAF, and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), a synthetic membrane-permeant diacylglycerol that activates protein kinase C selectively without triggering phosphoinositide hydrolysis (Lapetina *et al.*, 1985).

Because of the second-messenger activity that a diacylglycerol analogue of PAF might have, we set out to identify the PAF metabolite seen in our vascular smooth muscle cells and to study its metabolism and possible biological activity. We report here that smooth muscle cells convert small amounts of PAF to the diacylglycerol analogue 1-*O*-alkyl-2-acetyl-*sn*-glycerol (AAG). Synthetic AAG has previously been found to produce aggregation of rabbit platelets (Satouchi *et al.*, 1984), to stimulate differentiation of HL-60 cells to macrophage-like cells (McNamara *et al.*, 1984), and to produce in rats a hypotensive effect of significantly longer duration than that produced by PAF (Blank *et al.*, 1984a). AAG has also been shown to be a precursor for the biosynthesis of PAF in platelets (Blank *et al.*, 1984b; Satouchi *et al.*, 1984), but not in human umbilical vein endothelial cells (Blank *et al.*, 1986). In our studies, AAG appeared rapidly (<15 s) in the smooth muscle cells and persisted in the cellular lipids for ≥ 6 h. The formation of this diacylglycerol analogue and its persistence in cellular lipids suggest that it may play a role in the mitogenic effect of PAF on smooth muscle cells. Furthermore, because PAF may be released in proximity to endothelial and vascular smooth muscle cells by activated platelets, neutrophils, and macrophages, AAG may play a previously unrecognized role in intercellular communication in the vascular wall.

¹ Abbreviations: AAG, 1-*O*-alkyl-2-acetyl-*sn*-glycerol; 2-acyl-PAF, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; BSA, bovine serum albumin; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor).

Figure 1. Tracings of TLC scans showing distribution of radioactivity in cellular lipids after incubation with PAF. Smooth muscle cells were incubated for 15 s–1 min with 1 nM [^3H]-PAF in buffer containing 3.7 μM BSA. The incubation medium was removed, and ice-cold methanol was added to prevent further metabolism. The cells were then harvested by scraping; cellular lipids were extracted in chloroform:methanol and analyzed by TLC, with the use of a solvent system of chloroform:methanol:ammonia:water, 100:90:3:6. "X" indicates the unidentified metabolite, which comigrates with a diacylglycerol standard. Each panel represents 3 individual samples pooled for TLC analysis.

Results

Identification of AAG

Figure 1 shows a series of thin-layer chromatography (TLC) plate scans representing the distribution of radioactivity in smooth muscle cells after three very short incubations with 1 nM [^3H]-PAF. After 15 s, 38% of the intracellular radioactivity was found in an unidentified peak with an R_f of 0.9, designated as "X." Subsequently, the relative percentage of radioactivity contained in this metabolite decreased, although the total amount of radioactivity in peak "X" continued to increase as the incubation progressed because total uptake continued to increase. Metabolite "X" appeared before 2-acyl-PAF was detected in the chromatograms (bottom panel). Small amounts of this unidentified metabolite, representing 1–4% of the total intracellular radioactivity, were consistently found in all experiments. When the cells were incubated with 1 nM–10 μM PAF, a constant increase in PAF uptake was observed (data not shown), and the percentage of the radioactivity present in the form of Metabolite "X" remained constant.

Incubation of PAF with phospholipase C resulted in virtually complete hydrolysis of the PAF. When the reaction mixture was extracted with chloroform:methanol (2:1, v/v) and analyzed by TLC with the use of the basic solvent system, 96% of the radioactivity was contained in a single peak, with an R_f of 0.90. This product, prepared

by enzymatic hydrolysis, co-chromatographed in both basic and acidic TLC systems, with the unknown compound formed from PAF by the cells (Table 1). R_f values for both compounds in the two systems were similar to those of 1-stearoyl-2-arachidonyl glycerol and OAG, and were well separated from monoacylglycerol, phosphatidic acid, arachidonic acid, or PAF.

When this product was silylated in preparation for mass spectrometry, TLC analysis with the use

Table 1. Separation of unidentified metabolite and other compounds in two TLC systems

Compound	R_f Values	
	Acidic system*	Basic system†
Cellular metabolite	0.25	0.92
Phospholipase C product‡	0.25	0.92
1-Stearoyl-2-arachidonyl glycerol	0.29	0.92
1-Oleoyl-2-acetyl glycerol	0.20	0.89
Monoacylglycerol	0.10	0.81
Phosphatidic acid	0.18	0.03
Arachidonic acid	0.29	ND§
Metabolite A	0.11	0.81
PAF	0.00	0.16

* Mixed hexanes:ethyl ether:acetic acid:methanol, 170:40:4:4 (v/v).

† Chloroform:methanol:water:ammonia, 100:90:6:3 (v/v).

‡ Prepared by 1-h incubation of PAF with phospholipase C.

§ ND = not determined.

of the acidic solvent system revealed that >96% of the material was converted to a much less polar substance ($R_f = 0.63$ vs. 0.23 for the unsilylated compound). This change in migration is consistent with silylation of a free hydroxyl group and suggests that the PAF metabolite contains a free hydroxyl group. Although a scan of the TLC plate indicated that the silylated derivative was present as a single peak, this derivative was partially resolved into two overlapping peaks by gas-liquid chromatography (data not shown). This is consistent with the finding that hydrolysis of PAF by phospholipase C yields a mixture of 1,2- and 1,3-DAG isomers (Satouchi *et al.*, 1981).

Figure 2 shows a chemical ionization mass spectrum in ammonia of the trimethylsilyl derivative of the material prepared by hydrolysis of PAF with phospholipase C. The spectrum contains a protonated molecular ion ($M + 1$) of m/z 431, which is the molecular weight calculated for the TMS ether of both the 1,2- and 1,3- isomers of 1-*O*-hexadecyl-acetyl-*sn*-glycerol. The ion at m/z

448 represents the protonated molecular ion plus ammonia ($M + 1 + NH_3$). The large ion at m/z 90 is the TMS group itself; m/z 341 represents the molecular ion minus the TMS group; m/z 189 corresponds to $M - [O(CH_2)_{15}Me]$, m/z 376 represents $M - [Me_3Si] + NH_3$, and the ion at m/z 60 is the acetate group.

The metabolic product from a 2-h incubation of smooth muscle cells with PAF was purified, derivatized, and subjected to analysis by gas chromatography and mass spectrometry, with the use of chemical ionization with NH_3 . The very small amount of material extracted from the cells was not sufficient to produce a definitive mass spectrum. However, when the spectrum was scanned for the protonated molecular ion (m/z 431), there was a small ion with a retention time of 4:16 min. This agrees well with a retention time of 4:10 min for the m/z 431 ion observed in the standard shown in Figure 2, prepared by the phospholipase C treatment of PAF. Furthermore, a comparison of the two mass spectra from the

Figure 2. Mass spectrum of 1-*O*-alkyl-2-acetyl-*sn*-glycerol prepared by phospholipase C treatment of PAF. The AAG was silylated and assayed by GC/MS by the use of chemical ionization with NH_3 .

standard and the cellular metabolite reveals the same four ions in the low m/z range of the spectra (m/z 60, 72, 77, and 90) and three ions at the high m/z range (m/z 316, 431, and 448). Since both the ($M + 1$) ion m/z 431 and ($M + 1 + \text{NH}_3$) ion m/z 448 are present in the spectrum of the cellular product at levels slightly above background, it appears likely that the sample contained 1-*O*-hexadecyl-acetyl-*sn*-glycerol.

To obtain additional evidence for the identity of the metabolite, smooth muscle cells were incubated with 1-*O*-[^3H]-alkyl-PAF or 1-*O*-alkyl-2-[^3H]-acetyl-PAF. Figure 3 shows that the small amount of metabolite formed retained both the labeled alkyl group (middle panel) and the labeled acetyl group (bottom panel), and that it comigrated with the AAG standard prepared by hydrolysis of PAF with bacterial phospholipase C (top panel). As expected, neither lyso-PAF nor 2-acyl-PAF was detected in the incubation with the acetate-labeled PAF (bottom panel).

Metabolism of AAG

The uptake by smooth muscle cells of the AAG prepared by enzymatic hydrolysis of PAF was studied as the first step in examining its further metabolism. Cultures of smooth muscle cells were incubated with 1 nM [^3H]-PAF or the [^3H]-AAG prepared as described above. At intervals of 2–60 min, groups of four cultures were harvested and assayed for radioactivity. At all times tested, the smooth muscle cells took up the AAG to a much greater extent than PAF (Figure 4).

Unlike PAF, AAG was found to be a poor substrate for serum acetylhydrolase. When PAF was incubated at 37°C with buffer containing 0.25% fetal bovine serum, it was rapidly deacetylated to lyso-PAF, with a half-life of ~ 5 min (Figure 5). However, AAG was hydrolyzed much more slowly; approximately one-half of the radioactivity remained as unmetabolized AAG after 2 h. Even with a 10-fold higher concentration of serum, more than one-half of the radioactivity remained as AAG after 1 h. This stability of AAG in serum may contribute to its persistence in cellular lipids.

To study the metabolic fate of the incorporated AAG, a pulse-chase experiment was performed. Smooth muscle cells were incubated for 10 min with 1 nM [^3H]-AAG in buffer containing 2.5 μM bovine serum albumin (BSA). The incubation medium was then removed, the cultures were washed with BSA and buffer, and fresh 2.5- μM BSA solution was added. Groups of cultures were harvested at intervals and their cellular lipids extracted with chloroform:methanol and analyzed by TLC. At the end of the 10-min pulse, 26% of

Figure 3. Tracings of TLC scans showing distribution of radioactivity in cellular lipids after incubation with 1-*O*-[^3H]-alkyl-PAF or 1-*O*-alkyl-2-[^3H]-acetyl-PAF. Smooth muscle cells were incubated for 2 h with 0.2 μCi /well of either 1-*O*-[^3H]-alkyl-PAF or 1-*O*-alkyl-2-[^3H]-acetyl-PAF. The incubation medium was then removed, the cultures were washed with a buffer solution containing 2.5 μM BSA, and the cellular lipids were extracted and analyzed by TLC as described in Figure 1. Top panel, PAF and AAG standards. Middle panel, radioactive metabolites detected after incubation with 1-*O*-[^3H]-alkyl-PAF. Bottom panel, radioactive metabolites detected after incubation with 1-*O*-alkyl-2-[^3H]-acetyl-PAF. The prominent peak at approximately $R_f = 0.5$ seen in the bottom panel has not been identified, but this material also is present in the incubation with [^3H]-alkyl-PAF and in the chromatogram of the standards.

the incorporated radioactivity had already been converted to a metabolite. Within 1 h, 70% of the radioactivity was converted to metabolites; however, 6 h after the original pulse, 20% of the radioactivity remained in the cell as unmetabolized AAG.

Although the relative amounts of AAG and total metabolites did not change appreciably after 1 h, the pattern of metabolites did change over the 6-h incubation. Figure 6 shows a series of TLC plate scans. The top panel shows a single peak of AAG ($R_f = 0.90$) in the incubation medium. The second

Figure 4. Uptake of AAG and PAF by smooth muscle cells. Confluent cultures were incubated with 1 nM [³H]-PAF or [³H]-AAG in buffer with 2.5 μ M BSA. At intervals the incubation medium was removed from groups of 4 cultures; the cells were then washed with 50 μ M BSA and buffer and harvested by scraping into ice-cold methanol, and the cellular lipids extracted into chloroform:methanol. Aliquots of the chloroform:methanol extract were assayed for radioactivity. Each point represents the mean of 4 individual samples; the SEM was <4% for all points.

panel shows the cellular radioactivity at the end of the 10-min pulse, with a new radioactive peak at $R_f = 0.80$, designated as "A," representing a cellular metabolite of AAG (Table 1). The further redistribution of cellular radioactivity in AAG and its metabolites over the 6-h chase is seen in subsequent panels. The main metabolite of AAG, peak "A," continues to increase, but, at 30 min, two secondary metabolites begin to appear (broad peaks around $R_f = 0.37$ and 0.61 , designated as "B" and "C," respectively). At the end of the 6-h chase, approximately equal amounts of radioactivity are found in AAG, the main metabolite "A," and the two secondary metabolites "B" and "C." No attempt was made to identify these two secondary metabolites. At no time was any radioactivity detected in the area of the TLC plate where phosphatidic acid would be found ($R_f = 0.15$), suggesting that AAG is not a substrate for diacylglycerol kinase in the vascular smooth muscle cells.

Figure 7 shows a chemical ionization mass

spectrum of metabolite "A" extracted from smooth muscle cells. The large ion at m/z 461 represents the protonated molecular ion, which agrees with the molecular weight calculated for 1-O-alkyl-glycerol having a silyl ether on each of the two hydroxyl groups. The ion at m/z 478 represents the protonated molecular ion plus ammonia ($M + 1 + NH_3$), and the large ion at m/z 90 is the TMS group. As shown in Figure 6, this metabolite accumulates to higher levels than AAG over the course of a 2-h incubation. Also, it is more stable than AAG because it is not subject to acetyl migration. These factors probably account for the fact that enough of this product could be extracted to obtain a definitive mass spectrum.

Biological activity of AAG

To examine the possible effect of AAG on smooth muscle cell proliferation, AAG or PAF was added to synchronized cultures of confluent (Figure 8, top panel) or subconfluent (bottom panel) smooth muscle cells at various times before or after the addition of serum, which occurred at 0 h. PAF

Figure 5. Stability of PAF and AAG in fetal bovine serum. [³H]-PAF and [³H]-AAG were incubated at 37°C in buffer containing either 0.05% BSA or 0.25–2.5% fetal bovine serum. Aliquots were removed at intervals, extracted, and analyzed by TLC as described previously to determine the amount of radioactivity remaining as either PAF or AAG.

Figure 6. Tracings of TLC plate scans showing metabolism of [^3H]-AAG by smooth muscle cells after pulse labeling. Confluent cultures were incubated for 10 min with 1 nM [^3H]-AAG in buffer with 2.5 μM BSA. This medium was then removed, and the cultures were washed with 50 μM BSA and buffer. Fresh buffer solution containing 2.5 μM BSA was then placed in each well. After incubation, the medium was removed and the cultures were washed with BSA and buffer. The cells were then harvested by scraping into ice-cold methanol, cellular lipids were extracted with chloroform:methanol, and the distribution of radioactivity in the extracts was analyzed by TLC with the use of a solvent system of chloroform:methanol:water:ammonia (100:90:6:3, v/v). Each point represents 3 individual samples pooled for a single TLC analysis. The top panel shows the single peak of labeled AAG in the incubation medium at the beginning of the experiment. The second panel shows the distribution of radioactivity in the cell lipids at the end of the 10-min incubation with the [^3H]-AAG. Subsequent panels show the conversion of AAG radioactivity in the cells to a primary metabolite and 2 secondary metabolites at 15 min, 30 min, 1 h, and 6 h.

and AAG produced similar effects in both cases. Both compounds inhibited [^3H]-thymidine incorporation when they were added after the serum was added. However, in the confluent cultures, addition of either PAF or AAG up to 6 h before the serum was added resulted in a stimulation of [^3H]-thymidine incorporation relative to control cultures, as was seen previously (Stoll and Spector, 1989). No such stimulation was seen when the subconfluent cultures were incubated for up

to 6 h with either PAF or AAG before the serum was added.

Figure 9 shows that AAG is at least as effective as the physiologically occurring 1-stearoyl-2-arachidonylglycerol and the synthetic diacylglycerol analogue OAG in competing for phorbol ester binding sites in smooth muscle cells.

To obtain more direct evidence for AAG interaction with protein kinase C in these cells, AAG was substituted for diolein in an *in vitro* assay utilizing a homogenate of the smooth muscle cells (see Methods). Both AAG and OAG appeared to be as effective as diolein in the assay (data not shown); however, levels of protein kinase C activity in these smooth muscle cells were found to be very low. Therefore, this experiment was repeated with the use of a homogenate of NB41A3 neuroblastoma cells, which had previously been observed to have high levels of protein kinase C activity in an *in vitro* assay. Table 2 shows that AAG, OAG, and 1-stearoyl-2-arachidonyl-sn-glycerol are equally effective in substituting for diolein in the homogenate assay, indicating that AAG has the same capacity to activate protein kinase C as do the diacylglycerols. Addition of calcium and phosphatidylserine without diacylglycerol produced a level of activity intermediate between the control and the samples with added AAG or diacylglycerols. This stimulation produced in neuroblastoma cell homogenates suggests that the activation of protein kinase C by AAG is not restricted to a single cell type, such as the smooth muscle cells, but may be a more general response.

Binding of PAF to smooth muscle cells

To determine whether the proliferative response also may have resulted from a receptor-mediated process, a series of PAF binding experiments were performed. In three separate experiments, confluent cultures of porcine pulmonary artery smooth muscle cells were incubated with 25 pM–10 nM [^3H]-PAF for 10 min at 4°C, to minimize the incorporation and metabolism of PAF by the cells. In each experiment, the amount of [^3H]-PAF bound increased with increasing PAF concentration, but the ratio of PAF bound/free was constant over the entire concentration range. A typical experiment is shown in the top panel of Figure 10. When the experiment was repeated with bovine aortic smooth muscle cells, identical results were obtained (Figure 10, middle panel), indicating that both of these vascular smooth muscle cell lines lack the type of specific, high-affinity receptors for PAF observed in other systems. For example, a similar binding experiment was performed with

Figure 7. Chemical ionization mass spectrum of the trimethylsilyl ether derivative of 1-O-alkyl-glycerol extracted from smooth muscle cells. Cells were grown to confluence in T-150 flasks. Cultures were incubated with 10 μM PAF in serum-free growth medium containing 25 μM BSA for 2 h. The cells were then washed with buffer and harvested by scraping into ice-cold buffer. Cellular lipids were extracted with chloroform:methanol (2:1, v/v), and then separated by TLC by the use of Supelco 1 mm Silica Gel H preparative TLC plates. The band containing the unknown metabolite was scraped from the plate and extracted in chloroform:methanol, 1:1 (v/v). This material was then silylated and analyzed by GC/MS.

human platelets, a system in which PAF binding has been well documented. The platelet binding experiment was performed at room temperature to prevent platelet aggregation. When platelets were incubated with 50 pM–10 nM [^3H]-PAF (bottom panel of Figure 10), both specific and nonspecific binding of PAF were observed. The Scatchard analysis yielded a value of 988 high-affinity binding sites/platelet, which is similar to that reported by Valone *et al.* (1982) and Inarrea *et al.* (1984), and a K_a of 0.14 nM for the high-affinity component of binding.

Discussion

Several groups of investigators who have studied the binding of PAF to platelets (Valone *et al.*, 1982; Inarrea *et al.*, 1984) and neutrophils (Valone and Goetzl, 1983; Bussolino *et al.*, 1984) have reported the presence of two distinct classes of

binding sites. One of these is a high-affinity, low-capacity saturable class of sites present in very low numbers, ranging from an estimated 100–2000 sites per platelet to $\sim 28\,000$ sites per neutrophil. The other class of sites is nonsaturable, with an extremely large capacity for binding PAF. Hwang *et al.* (1983) have also identified specific, high-affinity binding sites for PAF on guinea pig smooth muscle membranes from ileum, trachea, and lung parenchyma. Our results with human platelets agree with these published findings. In addition, the nonsaturable uptake observed in both lines of vascular smooth muscle cells is consistent with the nonspecific, high-capacity PAF binding reported in other systems. However, while Hwang *et al.* were able to demonstrate specific, high-affinity PAF binding sites in membrane preparations from guinea pig visceral smooth muscle cells, no such high-affinity PAF binding sites were detected in cultured porcine or bovine

Figure 8. Timing of exposure of PAF or AAG affects $[^3\text{H}]$ -thymidine incorporation by smooth muscle cells. Cells were grown in 12-well plates; cultures were synchronized by a 24-h incubation in serum-free medium. PAF or AAG (10 nM) was added to synchronized cultures of confluent (top panel) or subconfluent (bottom panel) smooth muscle cells at the indicated times before or after the addition of serum. Control cultures were refed with fresh serum-free medium at the beginning of the experiment, but were not incubated with either PAF or AAG. At $t = 0$, serum (final concentration 1%) was added to all cultures, including controls. Beginning 16 h after the addition of serum, the cultures were incubated with 1 μCi $[^3\text{H}]$ -thymidine for 6 h, and the incorporation of radioactivity into trichloroacetic acid-precipitable cellular material was assayed. Data are expressed as percent of control values. Each bar represents the mean $\pm$ SEM of values obtained from 8 individual cultures.

vascular smooth muscle cells. This may represent a functional difference between vascular smooth muscle and other types of smooth muscle; alternatively, it may result from a loss of the high-affinity receptors in culture, or it may be a species difference. Because no high-affinity binding sites for PAF were observed in our smooth muscle

Figure 9. Inhibition of $[^3\text{H}]$ -phorbol-12,13-dibutyrate binding by AAG and diacylglycerols. Confluent smooth muscle cell cultures were washed with buffer and then incubated for 30 min at room temperature with buffer containing 0.5% dimethylsulfoxide, $[^3\text{H}]$ -phorbol dibutyrate ($[^3\text{H}]$ -PDBu), and increasing concentrations of 1-stearoyl-2-arachidonylglycerol (DAG), OAG, or AAG. After 30 min, the incubation medium was removed, the cells were washed with buffer and harvested, and aliquots were assayed for radioactivity. Each point represents the mean of values obtained from 4 individual cultures ($n = 6$ for controls); the SEM was $\leq 2\%$ for all values.

cells, it is likely that the proliferative response to PAF that occurs in these cells is produced by an alternate mechanism. One possibility is the formation of a diacylglycerol-like metabolite from PAF by the smooth muscle cells.

Table 2. Effect of diacylglycerols and AAG on protein kinase C activity in NB41A3 neuroblastoma cell homogenates*

Compound added	PKC activity (cpm)†
None	4 670 $\pm$ 570
Diolein	12 380 $\pm$ 680
AAG	13 770 $\pm$ 740
OAG	14 770 $\pm$ 1 630
1-Stearoyl-2-arachidonyl-glycerol	12 810 $\pm$ 1 620

* Values are mean $\pm$ SEM; $n = 4$.

† cpm γ - $[^{32}\text{P}]$ in phosphorylated histone III/10 μg protein. Cell homogenates were incubated for 5 min at 30°C with histone, γ - $[^{32}\text{P}]$ -ATP, phosphatidylserine, and 6 $\mu\text{g}/\text{ml}$ of diacylglycerol or AAG.

Figure 10. Binding of PAF by smooth muscle cells and platelets. Porcine pulmonary artery smooth muscle cells (PSMC) and bovine aortic smooth muscle cells (BASMC) were grown to confluency in 6-well plates. Cells were then incubated for 5 min at 4°C with ice-cold [3 H]-PAF (25 pM–10 nM) in buffer. After 5 min, the labeling medium was removed, and aliquots were assayed for unbound radioactivity. Cells were rinsed once with ice-cold buffer, harvested, and aliquots assayed for radioactivity. Human platelets (5×10^7 /sample) were incubated for 5 min at room temperature with [3 H]-PAF (50 pM–10 nM) in buffer (bottom panel). After 5 min, the incubation was stopped by centrifugation in a microcentrifuge. The supernatant was removed, and aliquots were assayed to determine unbound radioactivity. The pellet was washed once with buffer, recentrifuged, and then solubilized in 1% sodium dodecylsulfate at 37°C. Aliquots of the solubilized platelets were then assayed for radioactivity. Each point represents the mean of values obtained from 3 individual samples.

This possibility led us to investigate an unidentified PAF metabolite we had observed in our earlier studies of PAF metabolism in smooth muscle and endothelial cells. Based on its comigration with a diolein standard in two different TLC systems, the presence of molecular ions of 448 and 431 obtained by mass spectrometry of the cellular extract from smooth muscle cells, and the retention of the [3 H]-label in both the 1-*O*-alkyl and 2-acetyl groups, we believe that the Metabolite "X"

formed from PAF is 1-*O*-alkyl-2-acetyl-*sn*-glycerol. Satouchi *et al.* (1981) reported that when PAF was treated with phospholipase C, a mixture of 1,2- and 1,3-substituted glycerol isomers was produced. When we incubated PAF with phospholipase C and the products were converted to trimethylsilyl ether derivatives and analyzed by GC, two compounds separated with retention times of 4:06 and 4:13 min (data not shown). No attempt was made to determine whether the cellular metabolite is also a mixture of isomers, or whether the isomerization is an artifact of the digestion with bacterial phospholipase C or the subsequent extraction procedure. In any event, only the 1,2- isomer would be expected to be biologically active (Boni and Rando, 1985; Nomura *et al.*, 1986), since a free hydroxyl group in the 3-position is needed for binding and activation of protein kinase C (Ganong *et al.*, 1986). It is likely that AAG is formed in these cells by a phospholipase C, possibly the same enzyme that has been demonstrated to produce phosphoinositide hydrolysis in vascular smooth muscle cells (Takenawa, 1982; Greindling *et al.*, 1987). Since PDGF has been shown to activate phospholipase C (Williams, 1989), increased phospholipase C activity may be the basis for the apparent synergistic effect of PAF and PDGF on smooth muscle cell proliferation which we observed previously (Stoll and Spector, 1989).

The persistence of an intracellular diacylglycerol analogue derived from PAF may have important implications for the biological activity of PAF in vascular smooth muscle. Diacylglycerol second messengers generated by phosphoinositide hydrolysis are rapidly phosphorylated by diacylglycerol kinase to form phosphatidic acid. Therefore, diacylglycerols derived by this pathway have a half-life of only a few minutes (Bishop *et al.*, 1985; DeChaffoy de Courcelles *et al.*, 1985). However, no evidence for the phosphorylation of AAG was observed, suggesting that AAG is not a suitable substrate for diacylglycerol kinase. Furthermore, we have shown that AAG is relatively resistant to hydrolysis by serum acetylhydrolases, compared with PAF. Both of these factors may contribute to the persistence of AAG in cellular lipids. Therefore, AAG may remain in its unmodified and biologically active form for much longer periods than ordinary diacylglycerols. Since the concentration of AAG appears to be directly proportional to the amount of PAF taken up by the cells, it may accumulate in cells subjected to repeated exposure to even very low concentrations of PAF. It is clear that in the case of higher PAF concentrations, even 1% of total cellular uptake would represent a significant accumulation of AAG. Such

an accumulation could lead to a long-term activation of protein kinase C similar to that produced by phorbol esters (Castagna *et al.*, 1982).

Based on its migration in both acidic and basic TLC systems (Table 1) and on the chemical ionization mass spectrum (Figure 8), the primary metabolite of AAG appears to be a monoalkylglycerol. This is consistent with the finding that 1-alkyl-2-acetyl-*sn*-glycerol is converted to 1-alkyl-*sn*-glycerol by human umbilical vein endothelial cells (Blank *et al.*, 1986).

Our results suggesting that both AAG and PAF may either stimulate or inhibit smooth muscle cell proliferation, depending on the experimental protocol, are intriguing. Other investigators have observed similar apparently conflicting responses in other systems acting through the protein kinase C pathway. Nelson *et al.* (1988) have shown that phorbol esters can either stimulate or inhibit bradykinin-stimulated PAF production in porcine pulmonary artery endothelial cells, depending on the growth status of the cultures, the concentration of phorbol ester, and the time of incubation with phorbol esters relative to the bradykinin stimulus. Levin *et al.* (1988) have found that the time of incubation with phorbol myristate acetate is critical in determining its effect on the release of tissue plasminogen activator by human umbilical vein endothelial cells. Similarly, the effect of phorbol esters on thrombin-induced DNA synthesis in vascular smooth muscle cells (Huang *et al.*, 1988) and on the induction of the KC gene in porcine aortic endothelial cells (Shen *et al.*, 1988) and of *c-myc* and *c-fos* in 3T3 cells (Hall and Stiles, 1987) is dependent on the precise timing of the phorbol ester incubation relative to the stimulus. In these systems, the window in which the phorbol ester preincubation will stimulate the response rather than block or inhibit it ranges from 15 min to 2 hr. In addition, Majack and Goodman (1988) found that transforming growth factor- β stimulated smooth muscle cell proliferation in confluent cultures but was inhibitory in sparse cultures. In our system, variables such as degree of confluence, timing of preincubation with PAF or AAG relative to addition of serum, and time in G₀ may play a critical role in determining whether the activation of protein kinase C by a given concentration of PAF or AAG will result in a positive or negative proliferative response.

These findings suggest that PAF may act as an intercellular signal in the vascular wall through a mechanism that is independent of receptors. PAF is released by activated platelets (Blank *et al.*, 1984b), neutrophils (Camussi *et al.*, 1981; Ludwig *et al.*, 1984), and macrophages (Arnoux *et al.*, 1980; Roubin and Benveniste, 1985). Ca-

musi *et al.* (1983) have reported that PAF is released by cultured endothelial cells. By the use of bovine aortic endothelial cells grown on micro-pore filters, we have found that PAF is released by these cells into both the apical and interstitial fluid (unpublished results). The PAF released by subendothelial macrophages would be available directly to the smooth muscle cells; PAF released by platelets and neutrophils at the apical endothelial surface also might be available to the smooth muscle if the endothelium is damaged (Stoll and Spector, 1989). The present results suggest that PAF available to the target cells is taken up readily in a concentration-dependent manner, and that small amounts are converted by phospholipase C to AAG, a diacylglycerol analogue that persists in cellular lipids much longer than the diacylglycerol generated by the phosphatidylinositol cycle. This biologically active PAF metabolite then activates protein kinase C in the target cell, possibly on a long-term basis, and may thus initiate a cascade of events that affect multiple cellular processes, including proliferation. The activation of protein kinase C by AAG and subsequent cellular events are presently being investigated in our laboratory.

A unique aspect of this postulated signaling system would be that the second messenger generated by the external signal would be derived from the original signal molecule. Because the amount of AAG formed seems to be in direct proportion to the concentration of PAF available for uptake, the concentration of the messenger and, presumably, the magnitude of the response would be proportional to the amount of PAF in the local environment. Furthermore, because this signaling system would be independent of a receptor, it could coexist with a second receptor-mediated system in cells that had such receptors. Such a receptor-mediated system might control different responses, or it might act either antagonistically or synergistically with AAG. This might explain the observation that specific PAF antagonists, which have been shown to bind to PAF receptors, block some, but not all, of the biological responses to PAF in neutrophils (Henson, 1987).

Methods

Cell culture

Porcine pulmonary artery smooth muscle cells which had previously been characterized as a well differentiated smooth muscle cell line (Stoll and Spector, 1987) were grown in Dulbecco's Minimal Essential Medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), MEM Nonessential Amino Acids (GIBCO), MEM Vitamin Solution (GIBCO), 2 mM L-glutamine (Sigma), 15 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (Sigma, St.

Louis, MO), and 50 $\mu\text{g/ml}$ gentamicin (Schering Corp., Kenilworth, NJ). Cultures were grown at 37°C in a humidified atmosphere containing 5% CO₂. Stocks were subcultured weekly by trypsinization; for these experiments, the cells were used between passages 5 and 12. Bovine aortic smooth muscle cells used in the binding study were provided by Dr. Paul DiCorleto of the Cleveland Clinic Research Institute; they were cultured in the same manner as the porcine pulmonary artery smooth muscle cells. NB41A3 mouse neuroblastoma cells used in the protein kinase C assays were cultured in Ham's F10 medium supplemented with 15% horse serum, 2.5% fetal bovine serum, 100 U/ml of penicillin, 100 $\mu\text{g/ml}$ of streptomycin, and 294 $\mu\text{g/ml}$ of glutamine. Human platelets were obtained from a healthy adult volunteer in accordance with an approved Institutional Human Use Committee protocol, and were isolated and washed according to the procedure described by Czervionke *et al.* (1978). For the binding experiment, platelets were suspended in buffer at a concentration of 10⁸ platelets/ml.

Binding studies

PAF binding was determined by a modification of the method of Valone *et al.* (1982). Porcine pulmonary artery smooth muscle cells and bovine aortic smooth muscle cells were grown to confluence in 6-well plates. The cells were washed with ice-cold buffer; cells and all reagents were then chilled at 4°C for 20 min before binding experiments were performed. Smooth muscle cells were incubated for 5 min at 4°C with ice-cold [³H]-PAF (25 pM–10 nM) in buffer. After 5 min, the labeling medium was removed, and aliquots were assayed for unbound radioactivity. Cells were rinsed once with ice-cold buffer, and solubilized in 0.25 N NaOH at 37°C; aliquots were then transferred to scintillation vials and neutralized with 6 N HCl; 5 ml of Budget-Solve scintillation cocktail (Research Products International, Mt. Prospect, IL) was added to each vial, and the samples were assayed for radioactivity in a Beckman LS 2800 scintillation spectrometer. Quenching was monitored by channels ratio estimates. Human platelets were prepared as described under "Cell culture;" 5 × 10⁷ platelets/sample in Epindorf microcentrifuge tubes were incubated at room temperature with [³H]-PAF (50 pM to 10 nM) in buffer. After 5 min, the incubation was stopped by centrifugation for 2 min at full speed in a Beckman Microfuge 12 microcentrifuge. The supernatant was removed, and aliquots were assayed to determine unbound radioactivity. The pellet was washed once with buffer, recentrifuged, and solubilized in 1% sodium dodecylsulfate at 37°C. Aliquots of the solubilized platelets were then assayed for radioactivity.

Preparation of derivatives and mass spectrometry

PAF was obtained from Bachem (Basel, Switzerland). [³H]-PAF (1-O-[³H]-alkyl, mixture of C-16 and C-18 alkyl ethers, 81 Ci/mmol) was purchased from Amersham (Arlington Hts., IL). In separate incubations, both unlabeled and labeled PAF were hydrolyzed for 1 h at 37°C with phospholipase C (*Bacillus cereus*, Sigma, St. Louis, MO). After 1 h, the reaction mixture was extracted with chloroform:methanol (2:1, v/v). The lipid extract was subjected to TLC, with the use of a solvent system of chloroform:methanol:water:ammonia (100:90:6:3, v/v); to remove any remaining traces of PAF, the band corresponding to the product was scraped from the plate and extracted with chloroform:methanol, 1:1 (v/v). For mass spectrometry, the products of the phospholipase C incubation with PAF or the metabolite isolated from the cells were converted to the trimethylsilyl ether by incubation with bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane (Sylon BFT, Su-

pelco, Inc., Bellefonte, PA) in pyridine (Maas *et al.*, 1982). These trimethylsilyl derivatives were analyzed with a Riber R 10-10 quadropole mass spectrometer containing a 25 m × 0.2 mm gas-liquid chromatography column packed with 5% phenylmethylsilicone maintained at 260°C. The energy of the electron beam was 22.5 eV.

AAG incorporation and lipid analysis

For experiments measuring AAG uptake and subsequent metabolism, cultures were incubated with [³H]-AAG in DPBS containing 2.5 μM BSA. After incubation, the cells were washed thoroughly with 50 μM BSA followed by two washes with buffer solution to remove any adherent AAG before measurement of cell-associated radioactivity. Cells were then harvested by scraping into ice-cold methanol, and cellular lipids were extracted with chloroform:methanol, 2:1 (v/v). Aliquots of the lipid extract were placed in scintillation vials and evaporated to dryness; 5 ml of Budget Solve scintillation cocktail was added, and the samples were assayed for radioactivity in a Beckman LS 2800 scintillation spectrometer.

AAG and its metabolites were separated and analyzed by TLC. Aliquots of the chloroform:methanol extracts of cellular lipids were spotted on LK 60 Linear-K Silica Gel Plates (Whatman, Clifton, NJ) impregnated with 1.2% boric acid in 50% ethanol (Fine and Sprecher, 1982). In most experiments, separation was obtained with a solvent system of chloroform:methanol:ammonia:water (100:90:3:6, v/v). In some experiments, a second TLC system (mixed hexanes:ethyl ether:acetic acid:methanol, 170:40:4:4, v/v) was used for comparison. After development, the TLC plates were analyzed by a radio thin-layer chromatograph Model RS plate scanner (Radiomatic Instruments, Tampa, FL), which provides automatic peak search and integration.

Thymidine incorporation

Cell proliferation was measured by incorporation of [³H]-thymidine (Amersham) into trichloroacetic acid-precipitable material (DiCorleto and Bowen-Pope, 1983). Cultures of smooth muscle cells grown in 24-well plates were synchronized by serum starvation for 24 h. Experimental cultures were then incubated for 2 h with PAF in serum-free growth medium with 2.5 μM BSA, whereas controls were incubated with fresh serum-free medium. Serum was then added to the cultures to return the synchronized cells to the G₁ stage of the cell cycle. Sixteen to 18 h after the addition of serum, 1 $\mu\text{Ci/well}$ [³H]-thymidine was added as a single 4- to 6-h pulse to all wells. The medium was then removed, the cells were washed, and cold 5% trichloroacetic acid was added. The trichloroacetic acid-precipitable material was solubilized in 0.25 N NaOH for 1 h at 37°C, and an aliquot was transferred to a scintillation vial and neutralized with 6 N HCl; 5 ml of Budget Solve scintillation cocktail was added, and the radioactivity was measured in a Beckman LS 7000 scintillation spectrometer.

Synthesis of 1-O-alkyl-2-[³H]-acetyl-PAF

Acetate-labeled PAF was prepared from lyso-PAF by acetylation with [³H]-acetic anhydride (Amersham). Lyso-PAF (5 nmol) was dissolved in chloroform (2 ml), labeled acetic anhydride (7.5 nmoles) diluted with nonradioactive material and 7.5 nmol 4-dimethylaminopyridine (Sigma) were added, and the contents were stirred at room temperature for 48 h under nitrogen. After completion of the reaction, acetate-labeled PAF was separated and purified by preparative TLC with the use of chloroform:methanol/40% methylamine (60:36:5, v/v/v) as the solvent system.

Phorbol ester binding

1-oleoyl-2-acetyl-glycerol and stearoyl-2-arachidonyl-glycerol were obtained from Sigma. [^3H]-phorbol-12,13-dibutyrate (14.5 Ci/mmol) was obtained from Amersham. Phorbol ester binding was measured by incubating smooth muscle cell cultures for 30 min at room temperature with 2 nM [^3H]-phorbol-12,13-dibutyrate and 2–100 nM AAG, OAG (Sigma), or 1-stearoyl-2-arachidonyl-glycerol (Sigma) in buffer containing 0.5% dimethylsulfoxide. At the end of the incubation, the medium was removed, and the cells were washed once with buffer containing 50 μM BSA. The cells were then solubilized in 0.25 N NaOH at 37°C; aliquots were transferred to scintillation vials, neutralized with 6 N HCl, and assayed for radioactivity as described above.

Protein kinase C

Protein kinase C activity was assayed by a modification of the method of Kikkawa *et al.* (1983). Smooth muscle cells or NB41A3 neuroblastoma cells were grown to confluency in T-75 flasks; cells from two to three flasks were used for each assay. Cells were washed in buffer containing (in mM) 145 NaCl, 3 KCl, and 20 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.4, and then harvested by scraping into a small amount of 50 mM Tris-HCl, pH 7.4, containing 2 mM dithiothreitol and 2 mM ethyleneglycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid. The cell suspension was sonicated for 10 s and centrifuged for 1 h at 100,000 $\times$ g at 4°C in a Beckman ultracentrifuge. Aliquots (25 μl) of the supernatant were incubated for 5 min at 30°C with 1 mg/ml histone III-S (Sigma), 60 $\mu\text{g/ml}$ bovine brain phosphatidylserine (Avanti, Birmingham, AL), 25 μM ATP (Sigma), 0.1 $\mu\text{Ci/tube}$ γ -[^{32}P]-ATP (Amersham/Searle), and 6 $\mu\text{g/ml}$ diolein (Sigma) or one of the other diacylglycerol preparations; the total volume of the incubation mixture was 100 μl . After 5 min, a 50- μl aliquot of the reaction mixture was spotted on a P81 Whatman filter paper. The filters then were placed in Uni cassettes and washed in 75 mM phosphoric acid for 60 min with one change of phosphoric acid. The filters were placed in scintillation vials overnight in 0.5 ml NCS tissue solubilizer (Amersham/Searle); afterwards 10 ml Neutralizer Cocktail (Research Products International) was added, and the samples were assayed for radioactivity in the liquid scintillation spectrometer. Aliquots of a cell-free reaction mixture were spotted on filters, washed, and assayed as described; these blank values were subtracted from those obtained with the samples.

Acknowledgments

This work was supported by Arteriosclerosis Specialized Center of Research Grant HL 14230 from the National Heart, Lung and Blood Institute, National Institutes of Health, and by the Diabetes and Endocrinology Research Center Grant DK 25295 from the National Institutes of Health.

Received: August 3, 1989.

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