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Identification and pharmacological characterization of functional platelet-activating factor receptors on human B lymphoblastic cell lines

Travers, Jeffrey Bryant, Ph.D.
The Ohio State University, 1990
IDENTIFICATION AND PHARMACOLOGICAL CHARACTERIZATION
OF FUNCTIONAL PLATELET-ACTivating FACTOR RECEPTORS
ON HUMAN B LYMPHOBLASTIC CELL LINES

Dissertation
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By
Jeffrey Bryant Travers, B.S.

* * * * *

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<td>alkyl-lyso-phospholipid</td>
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<tr>
<td>AcylPAF</td>
<td>1-alkyl-2-acyl-GPC</td>
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<tr>
<td>$B_{\text{max}}$</td>
<td>maximal binding constant</td>
</tr>
<tr>
<td>BCDF</td>
<td>B-cell differentiation factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_i$</td>
<td>intracellular free calcium concentration</td>
</tr>
<tr>
<td>CS</td>
<td>calf serum</td>
</tr>
<tr>
<td>CPAF</td>
<td>carbamyl-PAF (1-alkyl-2-N-methyl carbamyl-GPC)</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3':5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CDP</td>
<td>cytosine diphosphate</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>ECL</td>
<td>equivalent chain length</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EBVDNA</td>
<td>Epstein-Barr viral DNA</td>
</tr>
<tr>
<td>F&lt;sub&gt;min&lt;/sub&gt; / F&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum/minimum fluorescence constant</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>G-protein</td>
<td>guanine nucleotide binding protein</td>
</tr>
<tr>
<td>GPC</td>
<td>glycero-3-phosphocholine</td>
</tr>
<tr>
<td>γ-IFN</td>
<td>gamma (immune) interferon</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salts solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethyl-piperazine-N’-2-ethane sulfonic acid</td>
</tr>
<tr>
<td>HETE</td>
<td>hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibition constant</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin 2</td>
</tr>
<tr>
<td>I&lt;sub&gt;1,4,5&lt;/sub&gt;P&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol 1,4,5, triphosphate</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>LGL</td>
<td>large granular lymphocytes</td>
</tr>
<tr>
<td>LTB&lt;sub&gt;4&lt;/sub&gt;</td>
<td>leukotriene B&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>LysoPAF</td>
<td>1-alkyl-2-lyso-GPC</td>
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<tr>
<td>m/z</td>
<td>mass/charge</td>
</tr>
<tr>
<td>NK cell</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethyl sulfonyl fluoride</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PHA (p)</td>
<td>phytohemagglutinin (type p)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PLA₂</td>
<td>phospholipase A₂</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet-activating factor (1-alkyl-2-acetyl-GPC)</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>SAC</td>
<td><em>Streptococcus A Cowan</em></td>
</tr>
<tr>
<td>S.D.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>tBDMS</td>
<td>N-methyl-N-(tertiary-butyldimethylsilyl) trifluoroacetamide</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>[³H]TdR</td>
<td>tritiated-thymidine</td>
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INTRODUCTION

A. THE HISTORY OF PAF

*Discovery and structural identification.* Platelet-activating factor (PAF) was the name given in the early 1970s by Jacque Benveniste to the unknown platelet aggregating factor(s) released from IgE-stimulated rabbit basophils [1,2]. In 1979-80, three groups [3-6] independently identified the structure of this platelet-aggregating substance as 1-alkyl-2(R)-acetyl-glycero-3-phosphocholine, the structure of which is shown in Figure 1. This phospholipid is a phosphocholine, with an ether-linked alkyl group consisting of 16 - 18 carbons at the sn-1 position, and an acetate moiety at the sn-2 position.

*Change in conception of action of phospholipids.* Before the discovery of this phospholipid 'platelet-activating factor', it was generally accepted that the biological effects of phospholipids were through non-specific interactions with cell membranes. The discovery that a phospholipid had biological activity (i.e, platelet aggregation) at picomolar concentrations shattered the concept that phospholipids exerted biological activities by mere 'physical' effects.

*Identification of biological activities.* Once the structure of PAF was determined, synthetic PAF became commercially available. This allowed further
Platelet-Activating Factor

Figure 1. The structure of platelet-activating factor (PAF)
investigations of the range of its biological activities. As was the case for eicosanoids, this 'platelet-activating factor' was found to have more biological activities than that for which it was first described. PAF has been shown to be involved in a myriad of pathophysiological processes ranging from platelet aggregation to parturition. PAF has also been shown to be synthesized and released from numerous tissues, and with the exception of erythrocytes [7], PAF has been found to be synthesized in every tissue in which it was examined. Several recent extensive reviews [8-11], and abstracts from more than five international scientific meetings, devoted exclusively to PAF, indicate the breadth and scope of study in this field.

*Other terms associated with PAF.* Although this factor has been shown to have more biological activities than just platelet aggregation/secretion, the term 'platelet-activating factor' has been kept for historical reasons. Other terms that have been found in the literature describing this novel class of biologically active phospholipids include AGEPC (alkyl-glycerol ether phosphatidylcholine) [6], APRL (antihypertensive polar renomedullary lipid) [12] and paf-acether [3]. In agreement with several recent conferences, the term 'platelet-activating factor' (PAF) will be used in this manuscript.

**B. PAF SYNTHESIS AND METABOLISM**

*The PAF metabolic cycle.* Even before the structure of PAF was fully
elucidated, it was known that its activity could be abolished by treatment with the enzyme phospholipase $A_2$ (PLA$_2$), which cleaves $sn$-2 substituents from phospholipids, producing the resultant lysophospholipid. With the structure of PAF known, it was shown that PAF's deacetylated derivative 1-alkyl-2-lyso-GPC (lysoPAF) was released along with PAF by stimulated leukocytes [13]. The finding that the chemical acetylation of lysoPAF resulted in biologically active PAF [13], also gave evidence that lysoPAF was a possible precursor/metabolite of PAF. Studies from the laboratory of Snyder [14] showed that a unique acetyltransferase enzyme catalyzed the acetylation of lysoPAF to PAF, utilizing acetyl-CoA as the source of acetate. It is now known that the synthesis and breakdown of PAF form a metabolic cycle as shown in Figure 2. PAF is 'stored' in cells as 1-alkyl-2-acyl-GPC. The acyl group may be one of several long chain fatty acids including arachidonate, linoleic, oleic, palmitate, and others [15]. Stimulation of the cell resulting in the activation of PLA$_2$ will cause production of lysoPAF and release of the long-chain fatty acid. The lysoPAF is then quickly acetylated to form PAF. Inasmuch as the $sn$-2 position of 1-alkyl-2-acyl-GPC is often enriched with arachidonic acid [15,16], the above stimuli could also result in the production of eicosanoids along with PAF. PAF is quickly broken down to lysoPAF by acetylhydrolase enzymes found intracellularly [17] and in serum [18,19]. The lysoPAF then formed is reacylated with a long-chain fatty acid (often arachidonic acid) to make 1-alkyl-2-acyl-GPC, thus forming a complete cycle (Figure 2).
Figure 2. The PAF metabolic cycle.
The cholinephosphotransferase pathway. Like other phosphatidylcholines (PCs), PAF can also be synthesized by a CDP-choline cholinephosphotransferase pathway. This 'de novo' pathway has been demonstrated in numerous tissues such as rat spleen [20], kidney homogenates [20], neutrophils [21] and chick embryo retinal tissue [22]. PAF is synthesized in this pathway by the transfer of a phosphocholine group to 1-alkyl-2(R)acetyl glycerol (AAG). The CDP-choline cholinephosphotransferase enzyme catalyzing this reaction is thought to be distinct from that involved in the conversion of diacylglycerol into PC [20]. Unlike the acetyltransferase enzyme, the cholinephosphotransferase enzyme appears to be independent of cellular stimulation, especially stimuli that result in increases in intracellular calcium levels [21,23].

C. THE PAF RECEPTOR

Evidence that PAF acts on a specific receptor. As shown in Figure 1, PAF is a chiral molecule. The demonstration that the unnatural (S) stereoisomer was relatively inactive [24], and that the naturally occurring (R) stereoisomer was active at low concentrations suggested that PAF exerted its effects through interaction with a specific receptor. Evidence for a specific PAF receptor has been obtained from radioligand binding studies using \([^3\text{H}]\text{PAF}\), and the development of compounds that block PAF-induced biological actions at doses that compete with PAF in binding studies (i.e., PAF receptor antagonists). \([^3\text{H}]\text{PAF}\) binding studies
Figure 3. The PAF receptor second-messenger systems in the platelet [31].
have demonstrated specific binding sites on such PAF-responsive tissues as platelets [25,26] neutrophils [27,28], monocytes [29], lung tissue [30] and others [31].

**Biochemical results of PAF receptor activation.** The PAF receptor is thought to be a plasma membrane protein [32,33] that may be coupled to transducing guanine nucleotide binding proteins [34,35]. The biochemical sequelae of the interaction of PAF with its receptor are outlined in Figure 3. In platelets [36-38] neutrophils [39,40] and monocytes [41] receptor binding results in activation of phospholipase C, leading to the phosphodiesteratic cleavage of inositol-containing phospholipids into water-soluble inositol polyphosphates and diacylglycerol. The inositol polyphosphate inositol 1,4,5 triphosphate (I\(_{1,4,5}P_3\)) induces calcium mobilization from intracellular (non-mitochondrial) stores [42]. Diacylglycerol (DAG) has been shown to activate Protein Kinase C (PKC) [43]. Thus, the interaction of PAF with its receptor in the above systems causes an increase in intracellular calcium levels ([Ca\(^{2+}\)]) and PKC activation. In platelets [44], neutrophils [45] and thyroid tissue [46] PAF receptor binding has been shown to inhibit adenylate cyclase. These cellular signals (calcium mobilization, PKC activation, adenylate cyclase inhibition) then mediate the response (aggregation, cellular activation, etc.) seen with PAF treatment.

**The mechanism of calcium mobilization.** PAF-induced intracellular calcium mobilization in platelets [47-49] neutrophils [49], monocytes [41,50] and PC-12
cells [51,52] is either partially or totally dependent upon the presence of extracellular calcium. This indicates that PAF may activate a calcium channel. PAF may activate this calcium channel directly, or through a second-messenger such as an inositol polyphosphate. Studies by Valone [53,54] showed that the calcium channel antagonists verapamil and diltiazam inhibited PAF-induced platelet aggregation at concentration which compete with PAF for binding to the platelet PAF receptor. These experimental findings were thought to provide indirect evidence that the PAF receptor is linked to a calcium channel. However, it is unlikely that the platelet PAF receptor is directly linked to a voltage-dependent calcium channel, as platelets are thought not to contain voltage-dependent calcium channels [55]. Further studies testing the effects of more potent voltage sensitive calcium channel blockers such as nimodipine [56] could probably yield more information concerning whether the effects of verapamil and diltiazem are related to their ability to block voltage-dependent calcium channels.

Properties of the PAF receptor. PAF receptor binding studies have been conducted using both whole cells and membrane preparations. The platelet PAF receptor has been the most studied. Binding studies with both intact cells and membrane preparations have indicated that there is a single class of binding sites (approximately 100-400 sites/cell) with a dissociation constant ($K_d$) of 1-3 nM [25,26,57-59]. In all cases there is agreement between the $K_d$ and the concentration of PAF needed to elicit half-maximal platelet activation (EC$_{50}$).
There are species differences in the number of PAF receptors, with rabbit platelets containing approximately 5 times more receptors per cell than human platelets [59]. In contrast, rat platelets do not contain PAF specific binding sites, and as expected, are not affected by PAF treatment [58].

Unlike the platelet PAF receptor, there is not good agreement concerning the characteristics of neutrophil PAF receptors. Unlike platelets, that do not metabolize PAF at concentrations below approximately 47 nM [31,57], neutrophils will readily metabolize PAF at concentrations used for binding [28,60]. Thus, neutrophil PAF binding studies are inherently more complex. Early studies of the neutrophil PAF receptor [26,27] which were conducted at room temperature did not yield consistent results, especially in the determination of receptor number. These discrepancies were probably due to artifacts introduced by ligand metabolism. Studies by Wykle and colleagues [28] indicate that $[^{3}H]$PAF is taken up but not metabolized by intact neutrophils at 4°C. Binding studies conducted at this temperature revealed that neutrophils express a high affinity binding site (2000 sites/cell) with $K_d = 200 \pm \text{ pM}$ [28]. This receptor number from binding studies at 4°C contrasts greatly to that found by Valone and Goetzl [27], whose binding studies conducted at room temperature indicated that neutrophils expressed $5 \times 10^6$ high affinity binding sites per cell. These discrepancies indicate the importance of ligand metabolism in PAF binding studies, since PAF metabolism will create significant binding artifacts.
There is also not good agreement as to the number of classes of neutrophil PAF binding sites. Several binding studies have supported the presence of both high and low affinity sites [28,61]. However, only the high affinity site is associated with a distinct function, indicating the possibility that the low affinity site may be due to a binding artifact such as non-receptor membrane uptake.

**PAF receptor subtypes.** Unlike other receptor types such as cholinergic, adrenergic, or histaminergic, little information exists as to the presence of different classes of PAF receptors based on the use of receptor antagonists. However, recent studies examining the effect of different PAF receptor antagonists on blocking PAF-induced effects in neutrophils and platelets suggest that there may be differences between the receptors on these different cell types [62-64].

**D. PAF RECEPTOR ANTAGONISTS**

Once the structure of PAF was elucidated, compounds with similar structure were synthesized and tested for PAF agonistic or antagonistic qualities. The first compound shown to act as a PAF receptor antagonist was CV-3988, developed by Takeda Chemical Industries, Ltd. [65]. Other PAF receptor antagonists were soon described. Among the newer PAF receptor antagonists is BN52021 [66], which was extracted from a Chinese plant (*Ginkgo biloba*) that had been found to have medicinal qualities. Unlike CV-3988, BN52021 does not structurally resemble PAF (Figure 4). Other compounds with anti-PAF activities include amiloride
derivatives [67] and the triazolobenzodiazepines triazolam and alprazolam [68,69]. The finding that these benzodiazepines inhibited the PAF receptor produced much excitement, as it was thought that PAF may be an endogenous ligand for the benzodiazepine binding site. However, the demonstration that other benzodiazepines such as diazepam and adinazolam did not have significant anti-PAF activity [70], indicated that the PAF receptor and the benzodiazepine binding site are separate entities.

**PAF receptor antagonist classes.** PAF receptor antagonists can be divided into two classes, those which are structural analogs of PAF, and those which bear little resemblance to native PAF. As shown in Figure 4, examples of structural analogs include CV-3988 [65], CV-6209 [71] and Ro19-3704 [72]. Compounds which have PAF receptor antagonistic qualities, but do not resemble PAF structurally, include BN52021 [66] and the triazolobenzodiazepines alprazolam and triazolam [68,69]. The concentrations of these compounds necessary to inhibit PAF effects is similar to the concentrations needed to compete with native PAF for binding to its receptor, suggesting that these compounds act by competing with PAF for binding to the PAF receptor.

**Physiologic effects of PAF and the systems used to evaluate PAF activity.** Compounds are tested for PAF agonistic and antagonistic qualities using both in vitro and in vivo assay systems. Depending on the cell type, PAF treatment in vitro can result in a variety of responses including aggregation, chemotaxis,
Figure 4. PAF receptor antagonists.
granule (and enzyme) release, increased phagocytosis and others [71]. Because of its ease and reproducibility, and the discovery of PAF receptors on platelets, platelet aggregation is often used as a screening assay for the testing of compounds for PAF-like and anti-PAF activities. Inasmuch as platelet aggregation is a very complex process [11], the finding that a compound inhibits PAF-induced platelet aggregation would not necessarily indicate receptor antagonism.

Intravenous PAF injections have been shown to result in many of the same pulmonary and circulatory alterations seen during IgE anaphylaxis [73]. These include thrombocytopenia, neutropenia, bronchoconstriction, hemoconcentration, cardiac dysfunction, pulmonary hypertension and edema [71]. Many of these pathophysiological effects do not have the sensitivity or reproducibility, or dose-dependence which would allow them to be used as parameters in an animal model system to test for PAF-like and anti-PAF activities. The in vivo responses that are most used are hypotension, hemoconcentration and bronchoconstriction [71]. In fact, the first PAF receptor antagonist discovered, CV-3988, was initially described as an inhibitor of PAF-induced hypotension in the rat [65].

*Problems in interpreting results of studies with PAF agonists and antagonists.*

Care must be exercised in the conclusions drawn from the testing of compounds for PAF receptor antagonistic qualities in both in vitro and in vivo studies. Many compounds may inhibit PAF effects by mechanisms other than competition with a PAF receptor. For example, as mentioned above, CV-3988 and other PAF
receptor antagonists have been shown to inhibit PAF-induced hypotension in the rat. However, other agents such as glucocorticoids [74] and thyrotropin-releasing hormone [75] also effectively inhibit PAF-induced hypotension and anaphylaxis. Obviously, these compounds do not act by competition with native PAF for binding to a PAF receptor. Similarly, treatment of platelets with agents that increase intracellular cAMP such as prostacyclin [76-78], prostaglandins of the E series [77], vasoactive intestinal peptide [76], or forskolin [77] will inhibit PAF-induced platelet aggregation.

Care must also be exercised in the conclusions drawn from the use of known PAF receptor antagonists in both in vitro and in vivo studies. It would be a very naive fellow indeed, who would attribute all of the clinical effects of triazolobenzodiazepines to their ability to compete with PAF for binding to the PAF receptor. In addition, several PAF receptor antagonists such as CV-3988 have been shown to inhibit PAF metabolism [79,80], and this same receptor antagonist, at high doses, even acts as a PAF agonist [71].

E. THE ROLE OF PAF IN IMMUNE CELL FUNCTION

Although PAF is synthesized in almost every tissue and is thought to be involved in processes that range from implantation to parturition, and platelet aggregation to pituitary secretion, much of PAF research revolves around the role of PAF as an immune cell modulator. These effects of PAF on granulocytes,
monocytes and lymphocytes are described below.

**PAF and granulocytes.** PAF was first described as a platelet aggregating agent released by stimulated granulocytic leukocytes [1,2]. Neutrophils and other granulocytic cells have been shown to synthesize PAF by both the acetyltransferase and the 'de novo' pathways [81-83]. Neutrophils express PAF receptors [26-28], and PAF treatment has been shown to result in intracellular calcium mobilization [39,84], PI hydrolysis [39,40], inhibition of adenylate cyclase [45], aggregation [39] and lysozyme release [39]. PAF and the PAF agonist CPAF (1-alkyl-2-N-methylcarbamyl-GPC) induce neutrophils to synthesize PAF [85] and leukotrienes such as leukotriene B\textsubscript{4} [85,86]. Inasmuch as these compounds can attract more leukocytes through their chemotactic properties [87], PAF would be expected to increase the inflammatory response with respect to granulocytes.

**PAF and monocytes.** PAF is synthesized by monocytic cells [83,88,89] and several monocytic cell lines have been shown to contain PAF receptors [29,41]. PAF treatment causes PI hydrolysis in a monocytic cell line [41], and intracellular calcium mobilization in both monocytic cell lines [29,41] and normal macrophages [50]. PAF affects monocytic function by increasing phagocytic ability [90-92], glucose utilization [92], and enhancing macrophage cytotoxicity [93]. Since PAF treatment additionally induces synthesis of leukotriene B\textsubscript{4} and peptidoleukotrienes [94], PAF would also be expected to increase the inflammatory response with respect to monocytes.
**PAF and lymphocytes.** Unlike neutrophils and monocytes, little is known about the role of PAF in lymphocyte function. Initial studies using lymphocytes indicated that they did not synthesize PAF, although stimulation with the ionophore A23187 resulted in production of lyso-PAF, suggesting a possible lack of the enzyme acetyl transferase [83]. More recent studies have shown that lymphocytes can synthesize or activate acetyl transferase and synthesize PAF [95-97]. Several leukemia cell lines of T- and B-cell origin (including MOLT-4 and Raji) have been shown to release a PAF-like material upon stimulation with the mitogenic lectin phytohemagglutinin (PHA) and the ionophore A23187 [95]. Although PAF is synthesized by lymphocytes, the effects of PAF on T-lymphocyte function have not been firmly established. In three-day cultures of lymphocytes and macrophages (peripheral blood mononuclear cells; PBMC) stimulated with a mitogenic lectin, PAF has been shown to inhibit T-lymphocyte proliferation at nanomolar [98] and micromolar [99] levels. Other studies have indicated that PAF is stimulatory [100] or has no effect [101] on mitogen-stimulated T-lymphocyte proliferation. Studies in the laboratory of Benveniste indicate that micromolar concentrations of PAF have direct effects on T-lymphocytes, down-regulating CD2 and CD3 cell markers in a time- and dose-dependent manner, but having no effect on HLA class I antigen expression [102]. Unfortunately, these studies did not utilize PAF receptor antagonists which could provide evidence that the effects of these relatively high doses of PAF were mediated by interaction with a specific receptor. Little is
known about the effects of PAF on B-lymphocyte function [103], and it is not known whether lymphocytes have PAF receptors.

In summary, both granulocytic and monocytic cells have been shown to be not only endogenous producers of PAF, but are also PAF-responsive. Although PAF has been described as playing a role in lymphocyte function, the majority of model systems used to examine the effects of PAF on lymphocyte function contained non-lymphocytic cells such as monocytes and granulocytes. These PAF-responsive cells would probably make an assessment of the effect(s) of PAF on lymphocytes very difficult. Unlike platelets [25,26,57-59] neutrophils [26-28] and monocytes [29,41], it is not known whether lymphocytes contain PAF receptors. Identification of a functional lymphocyte PAF receptor would be an important first step in the delineation of the role of this phospholipid in lymphocyte function.

F. HYPOTHESIS

Although there is evidence for a role of PAF in lymphocyte function, many questions remained unanswered. Several fundamental questions that need to be addressed include the characterization of PAF metabolism and identification/characterization of a possible lymphocyte PAF receptor. The hypotheses which were tested in this dissertation were: 1) lymphocytes can metabolize PAF; 2) lymphocytes express PAF receptors; and 3) lymphocyte function can be modulated by physiologic PAF concentrations.
METHODS

A. CELL CULTURE TECHNIQUES

1. MATERIALS

Cell culture medium supplements

Fetal bovine serum (FBS) and bovine calf serum (CS) were obtained from either Hyclone (Logan, Utah) or GIBCO (Grand Island, NY). All serum was heat-inactivated in a 56 °C water bath for 30 min prior to use. Penicillin and streptomycin were purchased from The Ohio State University Cell Culture Service. L-glutamine was obtained as a 200 mM 100 x stock solution from either GIBCO or Sigma Chemical Co, St. Louis, MO.

Complete cell culture medium

The complete cell culture medium consisted of Roswell Park Memorial Institute 1640 medium (RPMI 1640) (GIBCO) supplemented with 10 % FBS or CS, 25 mM N-2-hydroxyethyl-piperazine-N’-2-ethane sulfonic acid (HEPES), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. As L-glutamine has a limited shelf life once diluted, additional L-glutamine (approximately 2 mM) was added to any media that was more than one month old.
Balanced salt solutions

Phosphate buffered saline (PBS) was obtained from OSU Cell Culture Service.

A modified [28] Hank's Balanced Salt Solution (HBSS) was prepared at pH 7.4 in distilled water as follows:

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<tr>
<td>NaCl</td>
<td>8.0 g/l</td>
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<tr>
<td>KCl</td>
<td>0.4 g/l</td>
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<tr>
<td>Na₂HPO₄</td>
<td>0.09 g/l</td>
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<tr>
<td>KH₂PO₄</td>
<td>0.06 g/l</td>
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<tr>
<td>CaCl₂·2H₂O</td>
<td>0.206 g/l</td>
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<tr>
<td>MgCl₂·6H₂O</td>
<td>0.142 g/l</td>
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<tr>
<td>MgSO₄·7H₂O</td>
<td>0.10 g/l</td>
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<tr>
<td>D-glucose</td>
<td>1.0 g/l</td>
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<tr>
<td>NaHCO₃</td>
<td>0.35 g/l</td>
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For the experiments utilizing fura-2, 25 mM HEPES was used instead of NaHCO₃.

2. METHODS

Culture and maintenance of lymphocyte cell lines

Thirteen lymphoid cell lines were used in these studies. The characteristics of each cell line are shown in Table 6. MOLT-4, Raji, Daudi and CCRF-CEM were provided by The Ohio State University Cell Culture Service and were originally obtained from American Type Culture Collection (ATCC) Rockville, MD. Dakiki and NALM-6 were kindly provided by M.S. O'Dorisio, M.D. Ph.D., Children's Hospital, Columbus, OH.; BJAB, BJA/HR-1, P3HR-1, B95-8, sfBT, PB-1 and CB-1 were provided by James Shaw, Ph.D., Ohio State University Department of
Medical Microbiology and Immunology. All cells were grown as stationary suspension cultures in complete cell culture medium (with either FBS or CS as indicated in Table 6). Cell lines were maintained in log-phase growth at 37 °C under a 5% CO₂ atmosphere. These cells were subcultured every three days by resuspending at a concentration of 5 x 10⁵ viable cells/ml. This was accomplished by either removal of an appropriate volume of cell suspension with subsequent addition of fresh medium or by collection of cells by centrifugation and complete replacement of culture medium.

Isolation and purification of normal human peripheral blood mononuclear cells (PBMC)

Peripheral venous blood was obtained from healthy male adult volunteers by venipuncture and collected in sterile vacutainer tubes containing sodium heparin. Alternatively, leukocyte suspensions (buffy coats) were obtained from The American Red Cross. PBMC were isolated by density gradient centrifugation [103]. Approximately 15 ml of blood (or 5 ml of buffy coat) was transferred to a 50 ml sterile polystyrene centrifuge tube and diluted with 30 ml PBS. Fifteen ml of Ficoll-Paque (Pharmacia, Piscataway N.J.) or Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO.) was layered under the blood with a sterile aspirating needle and a 30 cc disposable syringe.

The tube was then centrifuged at 400 x g (approximately 1400 rpm) for 30 minutes at 18-20 °C using a Beckman J-6B centrifuge. Centrifugation created four
distinct layers within the tube. From top to bottom these layers contained: (1) plasma and platelets; (2) lymphocytes and monocytes (PBMC); (3) Ficoll; and (4) erythrocytes and granulocytes. The upper layer was carefully discarded and the narrow PBMC layer was transferred to another 50 ml centrifuge tube. The PBMC were resuspended in PBS and the tube was centrifuged at approximately 90 x g for 10 min at 18-20 °C. The resulting supernatant was discarded and the PBMC pellet was washed and centrifuged at least twice more to ensure removal of contaminating platelets. The resultant final cellular pellet contained primarily PBMC and was resuspended in complete medium or medium without serum for further purification procedures.

Isolation and purification of normal human T and B cells from PBMC

T and B cells were purified from PBMC by negative selection using Lympho-Kwik isolation reagents (One Lambda, Los Angeles, CA). These commercially available reagents consist of monoclonal antibodies and complement and are produced at the University of California Los Angeles and distributed by One Lambda Inc., Los Angeles, CA.

T cell isolation using T Lympho-Kwik utilized a slight modification of the procedure established in the laboratory of Dr. Charles Orosz [104]. Briefly, 25 x 10^6 PBMC were pelleted by centrifugation in a 15 ml centrifuge tube. To the cell pellet, 1.6 ml of Lympho-Kwik was added, and the cells gently mixed by resuspension with a siliconized pasteur pipette. The cells were then incubated in
a 37 °C shaking water bath for 45 minutes. Because this reagent works by negative selection, it is important to make sure that no cellular clumps were allowed to form, as these would allow contaminating cells to remain 'hidden' from the reagent. To these ends, the cells were resuspended with a siliconized pasteur pipette every 15 minutes during the incubation.

After a 45 minute incubation, the cells were mixed well with a pipette and 0.4 ml of RPMI 1640 was layered over the cell preparation. The cells were then centrifuged at 1500 x g for 5 min at 18-20 °C. The supernatant fraction was carefully removed and the tube containing the cell pellet was inverted and the sides of the tube were washed carefully with RPMI 1640 or PBS to remove any cellular debris. The pellet was then resuspended in RPMI 1640 and the tube was centrifuged at approximately 200 x g for 10 min at 18-20 °C. The resultant supernatant was discarded and the lymphocyte pellet was washed and centrifuged at least twice more to ensure removal of reagent and cellular debris. The cells were resuspended in complete cell culture medium or HBSS depending upon whether they were to be cultured or used directly for fura-2 fluorescence measurements.

The purity of the resultant T lymphocyte population with respect to contaminating platelets, neutrophils and monocytes was assessed in two ways. First, Wright-Giemsa stain was used for morphological identification of contaminating neutrophils and platelets. Morphological studies indicated a lack of
polynucleated cells and a platelet contamination of less than 1 platelet/100 T-lymphocytes. Second, contamination with monocytes was assessed by a mitogenesis test. The purified T cells were incubated with a mitogenic dose (0.25 or 0.5 μg/ml) of PHA-p for 72 hours, and proliferation was detected by \([^3]H\)thymidine (\([^3]H\)TdR) incorporation. The purified populations of T-lymphocytes did not proliferate in response to mitogenic doses of PHA-p (this is in agreement with the findings of Clouse et al. [104]). Because antigen-presenting cells (such as monocytes) are needed to allow T-lymphocyte proliferation in response to mitogenic lectins [105], the absence of a mitogen-induced proliferative response indicated that there was no significant monocyte contamination.

The B cell isolation procedure using B Lympho-Kwik was taken directly from the instructions accompanying the isolation reagent. Briefly, 15-20 x 10^6 PBMC were pelleted by centrifugation in a 15 ml centrifuge tube. To the cell pellet, 0.8 ml of B Lympho-Kwik reagent 1 was added, and the cells gently mixed by resuspension with a siliconized pasteur pipette. The cells were then incubated in a 37 °C shaking water bath for 60 minutes. The cell suspension was gently resuspended every 15 min to remove cell clumps. After the 60 minute incubation, the cells were mixed well with a pipette and 0.2 ml of RPMI 1640 was layered over the cell preparation. The cells were then centrifuged at 2000 x g for 2 min at 18-20 °C. The resultant supernatant was discarded and the pellet was gently resuspended in 0.5 ml of B Lympho-Kwik reagent 2. The cells were then
centrifuged at 2000 x g for 2 min at 18-20 °C. The lymphocytes were washed twice with RPMI 1640 to ensure removal of reagent and cellular debris.

**Isolation and purification of human neutrophils**

Human neutrophils were isolated from buffy coats obtained from the American Red Cross using the procedure of McDonald and Sprecher [106]. Briefly, 50 ml of buffy coat (1 bag) was emptied into a siliconized 250 ml graduated cylinder. The bag was then rinsed out with approximately 50 ml of 0.15 M NaCl, which was added to the cylinder. Approximately 75 ml of 0.15 M NaCl was added to bring the total volume to 175 ml. To this cell suspension, 25 ml of 77 mM EDTA was added with gentle mixing. Next, 50 ml of 6 % (w/v) Dextran T-500 (Pharmacia, Piscataway NJ) made up in 0.15 M NaCl was added, and the cylinder was gently turned upside down to mix the contents. The cylinder was then allowed to stand undisturbed for approximately 45 min to allow erythrocyte sedimentation to occur. The upper phase (approximately 75 ml) was then removed and placed into 50 ml centrifuge tubes. The tubes were then centrifuged 700 x g for 15 min at 18 - 20 °C. The pellet was then dissolved in HBSS and the cells were loaded onto ficoll (30 ml cells over 15 ml ficoll). The tubes were then centrifuged at 400 x g (approximately 1400 rpm) for 30 minutes at 18-20 °C. As previously discussed in the PBMC isolation section, centrifugation created four distinct layers within the tube. The top three layers were removed, leaving the granulocyte and erythrocyte pellet. This pellet was resuspended in HBSS, transferred to another
centrifuge tube, and centrifuged again at 200 x g for 10 min at 18 - 20 °C. The resultant pellet was resuspended in 20 ml of hypotonic media (0.155 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) to lyse erythrocytes and immediately centrifuged. After centrifugation, the tube should have a white cellular pellet and a reddish supernatant. If there was significant reddish coloration of the pellet, the hypotonic lysis step was repeated. The supernatant fraction was discarded and the neutrophil pellet was washed with HBSS and centrifuged at least twice more to ensure removal of contaminating cells and cellular debris. The resultant final cellular pellet contained primarily neutrophils with less than 5 platelets per 100 neutrophils, and less than 1 mononuclear cell per 100 neutrophils as assessed by Wright-Giemsa stain. The PMNs were then resuspended in HBSS for use in binding studies.

**Counting and determination of cell viability**

Cell density was determined by use of a hemacytometer. Wright-Giemsa stain was used to routinely stain cells for morphological identification. Trypan blue dye was used to determine cell viability. The cell suspension to be counted was either diluted with RPMI 1640, HBSS, PBS or added directly with either stain. The cellular suspension plus dye was then loaded onto a Neubauer type hemacytometer and allowed to stand for several minutes. Each of the four large corner squares were counted. If the cell count was less than 25 cells/square, then both sides of the hemacytometer were counted. The cell concentration (cells/ml) in the original cell suspension was then calculated using the following equation:
# of cells counted x dilution factor x 10^4

# of corner squares counted

B. BIOCHEMICAL TECHNIQUES

1. MATERIALS

PAF, CPAF and phospholipid standards

Labeled PAF (1-O-[^3]H)octadecyl-2-acetyl-GPC, 132 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL. Unlabeled PAF was either purchased from Sigma Chemical Company, St. Louis MO (1-O-alkyl-2-acetyl-GPC, 1-O-hexadecyl-2-acetyl-GPC) or from Boehringer Mannheim, Indianapolis, IN. (1-O-octadecyl-2-acetyl-GPC). LysoPAF (1-O-hexadecyl-2-lyso-GPC) was purchased from Sigma. Labeled CPAF (1-[^3]H)alkyl-2-N-methylcarbamyl-GPC, 56.4 Ci/mmol) was purchased from New England Nuclear Products, Boston, Mass. Purity of above radioactive ligands was assessed by TLC as described elsewhere in Methods and was > 95%. Unlabeled CPAF was synthesized as outlined in Appendix I.

Phosphatidylcholine and phosphatidylethanolamine were kindly provided by Dr. Howard Sprecher (O.S.U.) and were originally obtained from Avanti Biochemicals Inc., Birmingham AL. [^3]H)alkyl-acetyl glycerol was produced by treatment of [^3]H]PAF with phospholipase C [107].
PAF receptor antagonists and calcium channel blockers

BN52021 was kindly provided by Dr. Nicolas Bazan, LSU Eye Center, New Orleans, LA. CV-3988 and CV-6209 were gifts from Dr. Y. Oka, Takeda Chemical Industries, Ltd., Osaka, Japan. Ro-193704 was generously provided by Dr. Peter Sorter, Hoffman-LaRoche, Inc., Nutley, N.J. Alprazolam was a gift from Upjohn Pharmaceuticals, Inc., Kalamazoo, MI.

The calcium channel blockers diltiazem, verapamil and nimodipine were generously provided by Dr. John Enyeart (O.S.U.). Diltiazem was purchased as the hydrochloride salt from Marion Laboratories, Kansas City, MO. Verapamil was also obtained as the hydrochloride salt from Knoll Pharmaceutical Co., Whippany, NJ. Nimodipine was obtained from Dr. Alexander Scriabine, Miles Institute of Preclinical Pharmacology, New Haven, CT.

Other reagents

Phospholipase C (Bacillus cereus) and fatty acid-free fraction V bovine serum albumin (BSA) were purchased from Sigma. Dimethyl sulfoxide (DMSO) was purchased from Aldrich Chemical Co., Milwaukee, WI. All other organic solvents used were of the highest quality commercially available.

2. METHODS

Measurement of [³H]PAF metabolism

[³H]PAF was adjusted to 2.6 Ci/mmole by addition of unlabeled PAF.
Lymphocytes (2.5 x 10^6/ml) were resuspended with a final volume of 0.95 ml of RPMI 1640 without serum. To the above cell suspension 50 μl of aqueous solution containing [3H]PAF complexed to BSA (2.5 mg/ml) was added, bringing the final concentration to 5 x 10^{-8} M. The incubation mixture was shaken gently at 37 °C. All reactions were terminated by addition of 3.75 ml chloroform/methanol (1:2 v/v) to the cell suspension and the lipids were extracted by the technique of Bligh and Dyer [108]. Briefly, after addition of the chloroform/methanol, the cell suspension was vortexed and left at 4 °C overnight. To this one-phase system, 1.25 ml of chloroform and 1.25 ml water were added with vortexing between each addition. This resulted in a two-phase system, with the water and methanol on top, and chloroform at the bottom. The tubes were centrifuged at 90 x g for 10 min at 4 °C to enhance the phase separation. The chloroform fraction was carefully transferred by use of a siliconized pasteur pipette to another siliconized tube. Recovery of tritium label in the chloroform fraction was always > 87 %, and was unaffected by any drug treatment. Solvents were removed by a stream of nitrogen and the lipids were resuspended in chloroform/methanol 2:1 (v/v). The phospholipids and phospholipid standards were separated on LK6D linear K silica gel TLC plates (Whatman Inc., Clifton, NJ.) using an acidic solvent system [11] containing chloroform/methanol/water/glacial acetic acid (65:35:8:1 v/v). The distribution of radiolabel was assayed by zonal scanning (2.5 - 5 mm increments). To each fraction 3 ml of
scintillation cocktail was added, and the radioactivity was measured in a Beckman LS6800 liquid scintillation counter.

**Measurement of [³H]PAF binding**

The [³H]PAF binding studies were conducted with slight modification of those used in the laboratories of Wykle and O'Flaherty [28] in quantitating human neutrophil PAF binding. Lymphocytes (5 x 10⁶ cells), were washed and resuspended to a final volume of 0.9 ml with HBSS in siliconized 15 x 85 mm glass tubes. Suspensions were then simultaneously exposed to 50 μl of aqueous solution containing [³H]PAF (or [³H]CPAF) complexed to BSA (10 mg/ml), plus either 50 μl unlabeled PAF, lysoPAF or other binding inhibitor complexed to BSA, or BSA alone. BN52021 and alprazolam were added in dimethyl sulfoxide (DMSO). DMSO at the final concentration used (0.2 %), did not affect PAF binding. The mixture of cells, [³H]PAF and inhibitors were shaken gently at 4 °C for the appropriate times. Each sample was run in triplicate. Suspensions were harvested by suction through GF/C filters (Whatman Inc., Clifton, NJ) premoistened with 10 mg/ml BSA using a Hoefer filtration apparatus (Hoefer Scientific, San Francisco, CA). The tubes were then washed three times with 5 ml of cold HBSS. The wells were then washed with 10 ml of additional HBSS. The filters were air-dried and placed in scintillation vials to which 4 ml of scintillation cocktail was added. The filters were allowed to stand in cocktail for at least eight hours before counting in a Beckman LS6800 scintillation counter that
was programmed to measure each sample's quench and determine disintegrations per minute (dpms) from counts per minute (cpms) utilizing tritium standards. The siliconized tubes retained essentially none of the \([\text{H}]\text{PAF}\) added. The BSA-treated filters retained about 1% of added \([\text{H}]\text{PAF}\). This binding was independent of incubation time, and amount of labeled or unlabeled PAF present. Samples were corrected for filter retention of radiolabel by running control samples that lacked cells. Specifically bound PAF was determined by incubation with excess (1 - 5 \(\mu\text{M}\)) unlabeled PAF. Scatchard analysis [109] of the data was carried out using analytical software (GraphPAD, San Diego, CA).

**Measurement of intracellular calcium concentrations**

To monitor intracellular calcium concentrations (\([\text{Ca}^{2+}]_i\)), the fluorescent calcium binding dye fura-2 was utilized. Fura-2 loading was achieved by incubating the cells (2.5 - 5 x 10^6 cells/ml) with 2 \(\mu\text{M}\) fura-2/AM (Molecular Probes, Inc. Eugene OR) for 10 min at room temperature, followed by a wash with HBSS containing 1.4 mM CaCl_2 and 25 mM HEPES, pH 7.4. Cells were then diluted to 1 x 10^6 cells/ml and post-incubated at room temperature for two hours to facilitate hydrolysis of the fura-2 ester. Prior to measurement, an aliquot (3 ml) of cells was transferred to a fluorimeter cuvette and prewarmed to 37 °C. Fluorescence signals were recorded in a Perkin-Elmer LS-5B spectrofluorimeter, at excitation and emission wavelengths of 340 nm and 510 nm, respectively. All experiments were performed at 37 °C with constant mixing. PAF, lysoPAF,
BN52021, alprazolam, CV6209, and other compounds were introduced with DMSO as the solvent. CV-3988 was introduced with ethanol as the solvent. The final concentration of solvent was < 0.3 %, which did not affect baseline $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ was calculated using the following equation [110]:

$$[\text{Ca}^{2+}]_i = K_d \times \left[ \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)} \right]$$

where $K_d$ is the apparent dissociation constant of fura-2-$\text{Ca}^{2+}$ and is 224 nM at 37 °C, $F$ is the fura-2 fluorescence measured during the experiments, and $F_{\text{max}}$ and $F_{\text{min}}$ are the fluorescence when fura-2 is calcium-saturated and calcium-free, respectively. At the end of each experiment, intracellular fura-2 was released by rupturing the cell membrane with digitonin. $F_{\text{max}}$ and $F_{\text{min}}$ were then determined in the presence of 1.5 mM $\text{Ca}^{2+}$ or excess EGTA at pH 8.

C. IMMUNOLOGICAL TECHNIQUES

1. MATERIALS

Lectin mitogen and cytokines

Phytohemagglutinin (P form) (PHA-p) was obtained from Sigma Chemical Company, St. Louis MO. The lyophilized PHA-p (5 mg) was rehydrated with 10 ml of sterile PBS to give a stock solution of 0.5 µg/µl. This stock solution was aliquoted and stored frozen at -70 °C and remained stable for approximately six
weeks.

Interleukin-2 (IL-2) used in the lymphocyte proliferation experiments outlined in Appendix II was obtained as delectinated PHA-induced human IL-2 (Cellular Products Inc., Buffalo, NY). This IL-2 was free from immune (gamma)-interferon and B cell growth factor contamination. Recombinant IL-2 used elsewhere, gamma-interferon (γ-IFN) and interleukin-1 alpha (IL-1α) were generously provided by Dr. George Cox, National Cancer Institute, Frederick MD.

**Bacterial lipopolysaccharide (LPS) and fibrinogen**

Bacterial lipopolysaccharide (LPS) from *E. coli* was purchased from Sigma Chemical Company, St. Louis MO. LPS stock (1 μg/μl) was made up by addition of 25 ml of PBS to 25 mg of LPS. This stock was aliquoted and stored frozen at -70 °C and was used within two months. Essentially plasmin(ogen)-free human fibrinogen was also purchased from Sigma. A stock of 10 mg/ml was created by addition of sterile RPMI 1640, then filtered with a 0.25 μm low protein-binding sterile filter. This fibrinogen was aliquoted and stored at -70 °C, and was used within two months.

**sfBT conditioned media**

Spent media from the tamarin (*Saguinus oedipus*) lymphoid cell line sfBT [111] was kindly provided by Dr. James Shaw (O.S.U.). This media was filtered with a 0.25 μm low protein-binding sterile filter and the pH was adjusted by addition of 25 mM HEPES (pH 7.3). Undiluted conditioned media (100% stock) was
aliquoted and stored at -70 °C, and was used within two months.

\[^{3}H\text{Thymidine}\]

\[^{3}H\text{Thymidine}\] (\[^{3}H\text{methyl-thymidine}, 6.7 \text{ Ci/mmol}\]) \(([^{3}H]\text{TdR})\) was obtained from New England Nuclear Research Products, Boston, MA. One ml of 1 mCi/ml of \[^{3}H\text{TdR}\] was diluted with 19 ml of sterile PBS to make a stock solution of 0.5 μCi/10 μl which was then stored at 4 °C. \[^{3}H\text{TdR}\] stock solutions were used within six months.

2. METHODS

Lymphocyte proliferation assay

Proliferation of both normal and transformed lymphocytes was detected by \[^{3}H\text{thymidine}\] (\[^{3}H\text{TdR}\]) incorporation [112]. Mitogen-induced PBMC proliferation assays were conducted with slight modifications of those established by Cox, et. al. [113]. Briefly, PBMC were resuspended at a concentration of 1 x 10⁶ viable cells/ml. Either the mitogenic lectin phytohemagglutinin (PHA-p) alone or mitogen plus other agents were added in triplicate to a flat-bottomed 96-well microtiter plate in a total volume of 100 μl of medium. One hundred μl of PBMC suspension (1 x 10⁵ cells/well) was then added to provide a final volume of 200 μl/well. The PBMC were then cultured for 72 hours at 37 °C in a humidified 5% CO₂ incubator. At this time, 10 μl (0.5 μCi) of \[^{3}H\text{TdR}\] was added to each microtiter well using a sterile repeating Hamilton syringe. The PBMC were then
incubated for an additional 16 hours (88 hour total incubation) and then harvested onto 934-AH glass fiber filters (Whatman Inc., Clifton, NJ) using an automated 24-well cell harvester (Brandel). The filters were then air-dried and placed into 6 ml scintillation vials, to which approximately 3 ml of scintillation cocktail was added. The filters were allowed to stand in cocktail for at least three hours before counting in a Beckman LS6800 scintillation counter that was programmed to measure each sample's quench and determine disintegrations per minute (dpm) from counts per minute (cpm) utilizing tritium standards. The resultant amount of $[^3]$H]TdR incorporated into cellular DNA was expressed as the mean dpm ± standard error of the mean (S.E.M.) after subtracting the background dpm (cells without PHA-p).

Proliferation of Raji lymphoblasts was assessed in a similar manner as PBMC, except the cells were seeded at an initial concentration of $1 \times 10^4$/well, and the $[^3]$H]TdR pulse was of eight hours duration. The results were also expressed as the mean dpm ± S.E.M., but background dpm were not subtracted.
RESULTS

A. THE METABOLISM OF PAF IN T-LYMPHOCYTES

*Incubation of $^3$H]PAF with MOLT-4*. In order to determine whether PAF is metabolized by T-lymphocytes, we first utilized a stable leukemia cell line of T-cell origin (MOLT-4) [114]. When 1-$[^3]$H}alkyl-2-acetyl-GPC was incubated with MOLT-4 lymphoblasts for 64 minutes and the products examined by TLC, a majority of the label (>60%) was found in a product migrating with 1-alkyl-2-acyl-GPC containing a long chain acyl group at the sn-2 position (Figure 5). The distribution of label in 1-alkyl-2-acetyl-GPC, 1-alkyl-2-lyso-GPC, 1-alkyl-2-acyl-GPC and neutral lipids as a function of time of incubation with MOLT-4 lymphoblasts is shown in Figure 6. At 64 minutes the following distribution of label was observed: 1-alkyl-2-lyso-GPC, 3%; 1-alkyl-2-acetyl-GPC, 17%; 1-alkyl-2-acyl-GPC, 67%; neutral lipid, 9%. As shown in Figure 7, when the cells and media were extracted separately, $[^3]$H]PAF was rapidly taken up by the cells and converted by deacylation-transacylation to 1-$[^3]$H}alkyl-2-acyl-GPC, which accumulated intracellularly. These studies indicated that MOLT-4 lymphoblasts can metabolize PAF.
FIGURE 5. DISTRIBUTION OF LABEL IN PRODUCTS AFTER INCUBATION OF 1-[\textsuperscript{3}H]ALKYL-2-ACETYL-GPC WITH MOLT-4 LYMPHOBLASTS.

1-[\textsuperscript{3}H]alkyl-2-acetylGPC (50 nM) was incubated with MOLT-4 lymphoblasts for 64 min at 37 °C, and the lipids were extracted as described in Methods. An aliquot of the labeled products was separated by TLC and radioassayed by zonal scanning (2.5mm increments). The peak numbers identify the following lipids: 1-alkyl-2-lyso-GPC (peak I, \(R_f=0.23\)); 1-alkyl-2-acetyl-GPC (peak II, \(R_f=0.32\)); 1-alkyl-2-acyl-GPC (peak III, \(R_f=0.46\)); unknown component that migrates slightly above phosphatidylethanolamine (\(R_f=0.64\)) (peak IV, \(R_f=0.71\)); Neutral lipid comigrating with 1-alkyl-2-acetyl glycerol (peak V). Addition of 1-[\textsuperscript{3}H]alkyl-2-acetyl-GPC followed rapidly by addition of 3.75ml of chloroform/methanol (2:1 v/v) resulted in >97% of label residing in peak II.
FIGURE 6. TIME COURSE OF THE METABOLISM OF 1-[3H]ALKYL-2-ACETYL-GPC IN MOLT-4 LYMPHOBLASTS.

1-[3H]alkyl-2-acetyl-GPC (50 nM) was incubated with MOLT-4 lymphoblasts for various times and the lipids extracted and separated as in Figure 5. The data are expressed as the percentage of total radioactivity found in 1-[3H]alkyl-2-acetyl-GPC (▲), 1-[3H]alkyl-2-lyso-GPC (□), 1-[3H]alkyl-2-acyl-GPC (●) and [3H]neutral lipid (■). The values are mean ± S.E.M. of 4-8 separate experiments utilizing duplicate samples.
FIGURE 7. TIME COURSE OF THE METABOLISM OF 1-[\textsuperscript{3}H]ALKYL-2-ACETYL-GPC IN MOLT-4 LYMPHOBLASTS; SEPARATION OF MEDIA AND CELL-ASSOCIATED METABOLITES.

1-[\textsuperscript{3}H]alkyl-2-acetyl-GPC (50 nM) was incubated with MOLT-4 lymphoblasts for various times and the cells and media separated by centrifugation. The lipids in the media (A) or the cells (B) were then extracted and separated as in Figure 5. The data are expressed as the percentage of total radioactivity found in 1-[\textsuperscript{3}H]alkyl-2-acetyl-GPC (\textbullet{}), 1-[\textsuperscript{3}H]alkyl-2-lyso-GPC (\textcircled{A}), 1-[\textsuperscript{3}H]alkyl-2-acyl-GPC (\textcircled{O}) and [\textsuperscript{3}H]neutral lipid (■). The values are mean ± S.E.M. of 3 separate experiments utilizing duplicate samples.
FIGURE 8. TIME COURSE OF THE METABOLISM OF 1-[³H]ALKYL-2-ACETYL-GPC IN PERIPHERAL BLOOD T LYMPHOCYTES.

1-[³H]alkyl-2-acetyl-GPC (50 nM) was incubated with peripheral blood T lymphocytes, purified as described in Methods, for various times and the lipids were then extracted and separated as in Figure 5. The data are expressed as the percentage of total radioactivity found in 1-[³H]alkyl-2-acetyl-GPC (△), 1-[³H]alkyl-2-lyso-GPC (○), 1-[³H]alkyl-2-acyl-GPC (●) and [³H]neutral lipid (■). The values are mean ± S.E.M. of 2 separate experiments utilizing duplicate samples.
Incubation of \[^3\text{H}\]PAF with peripheral blood T-lymphocytes. The metabolism of \[^3\text{H}\]PAF was then examined in normal human peripheral blood T-lymphocytes purified using the T Lympho-kwik method. The distribution of label in peripheral blood T-lymphocytes as a function of time is shown in Figure 8. The pattern of PAF catabolism in these cells is similar to the MOLT-4 cell line, but the rate is much slower. At 96 minutes the following distribution of label was found: 1-alkyl-2-lyso-GPC, 8%; 1-alkyl-2-acetyl-GPC, 55%; 1-alkyl-2-acyl-GPC, 32%; neutral lipid, 5%. The products shown in Figures 5, 6 and 8 were extracted from the complete incubation mixture and thus represent products derived from both cells and medium. Incubations of 1-[\[^3\text{H}\]alkyl-2-acetyl-GPC under standard conditions in the absence of lymphocytes resulted in no appreciable catabolism. The metabolism of 1-[\[^3\text{H}\]alkyl-2-acetyl-GPC was temperature dependent with >90% of label residing in PAF after 64 minute incubations at 4 °C (data not shown). These experiments indicated that normal human T-lymphocytes can metabolize PAF.

B. THE EFFECT OF PAF RECEPTOR ANTAGONISTS AND PMSF ON MOLT-4 PAF METABOLISM

The effect of PAF receptor antagonists on PAF metabolism. Since the PAF receptor antagonists BN52021 and CV-3988 have been shown to inhibit PAF metabolism in intact platelets [79,80], we determined the effect of the PAF
FIGURE 9. THE EFFECT OF PAF RECEPTOR ANTAGONISTS ON MOLT-4 PAF METABOLISM.

PAF receptor antagonists CV-3988 (○), Ro19-3704 (●), BN52021 (□) or vehicle (ethanol or DMSO) control (■) were incubated with MOLT-4 lymphoblasts for 5 min before addition of $[^3H]$PAF. The data are expressed as the percentage of total radioactivity recovered (relative to control) as 1-alkyl-2-acyl-GPC following a 16 min incubation of MOLT-4 lymphoblasts with 1-$[^3H]$alkyl-2-acetyl-GPC (50 nM) using the standard conditions described in Figure 5. The values are mean ± S.E.M. of 3 separate experiments utilizing duplicate samples.
receptor antagonists CV-3988, Ro19-3704 and BN52021 on PAF metabolism in MOLT-4 cells. CV3988 and Ro19-3704, which structurally resemble PAF, inhibited MOLT-4 PAF metabolism in a dose-dependent manner (Figure 9). However, BN52021 (up to 200 μM) had no effect on MOLT-4 PAF metabolism. None of the PAF receptor antagonists at the concentrations used affected the viability of the MOLT-4 cells as measured by trypan blue exclusion. These studies indicate that MOLT-4 lymphoblast PAF metabolism is different from that of platelets with respect to the ability of BN52021 to inhibit it.

Effect of PMSF on [³H]PAF metabolism in MOLT-4. The serine hydrolase inhibitor phenylmethylsulfonyl fluoride (PMSF) has been shown to inhibit the metabolism of 1-[³H]alkyl-2-acetyl-GPC in rabbit [115] and human [116] platelets, and in neonatal rat myocytes [117]. In order to determine whether lymphocyte PAF metabolism was affected by PMSF, we preincubated 1 mM PMSF with MOLT-4 cultures before addition of [³H]PAF as described in Table 1. PMSF (1 mM) inhibited the metabolism of 1-[³H]alkyl-2-acetyl-GPC in MOLT-4 lymphoblasts as shown in Table 1. The metabolic inhibition by 1 mM PMSF was not accompanied by a decrease in cell viability as measured by trypan blue dye exclusion. These results indicated that PAF metabolism in T-lymphocytes and MOLT-4 lymphoblasts is similar to that found in platelets and neonatal rat myocytes with regard to PMSF inhibition.
Table 1. INHIBITION BY PHENYL METHYL SULFONYL FLUORIDE OF THE METABOLISM OF 1-[^3]HALKYL-2-ACETYL-GPC

MOLT-4 lymphoblasts were prepared as indicated in Methods and pretreated with 1 mM phenylmethyl sulfonyl fluoride (PMSF) or its solvent DMSO (0.2% final concentration) at 37 °C for 15 minutes. The cells were then washed and resuspended in fresh RPMI 1640. 1-[^3]Halkyl-2-acetyl-GPC (50 nM) was then incubated with cells and metabolites analyzed as indicated in Figure 5. Each point represents the mean ± S.E.M. of three separate experiments utilizing duplicate samples.
Table 1. INHIBITION BY PHENILMETHYL SULFONYL FLUORIDE OF THE METABOLISM OF 1-[³H]ALKYL-2-ACETYL-GPC

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<td></td>
<td>1-Alkyl-2-lyso-GPC</td>
<td>1-Alkyl-2-acetyl-GPC</td>
</tr>
<tr>
<td>Control</td>
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<td>50.6 ± 7.7</td>
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<td>64</td>
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<td>42.8 ± 2.3</td>
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</table>
FIGURE 10. TIME COURSE OF THE METABOLISM OF 1-[³H]ALKYL-2-ACETYL-GPC IN RAJI LYMPHOBLASTS.

1-[³H]alkyl-2-acetyl-GPC (50 nM) was incubated with Raji lymphoblasts for various times and the lipids extracted and separated as in Figure 5. The data are expressed as the percentage of total radioactivity found in 1-[³H]alkyl-2-acetyl-GPC (△), 1-[³H]alkyl-2-lyso-GPC (○), 1-[³H]alkyl-2-acyl-GPC (○) and [³H]neutral lipid (□). The values are mean ± S.E.M. of 3 separate experiments utilizing duplicate samples.
C. THE METABOLISM OF PAF AND CPAF IN RAJI B-LYMPHOBLASTS

*The metabolism of PAF in Raji cells.* The previous experiments suggested that human T-lymphocytes metabolized PAF by the deacetylation-reacylation pathway found in other cell types such as neutrophils and platelets [16,60,118,119]. We next examined PAF metabolism in Raji, a B-cell line established from a patient with a Burkitt lymphoma [120]. As shown in Figure 10, Raji lymphoblasts readily metabolized PAF, forming 1-alkyl-2-acyl-GPC with a transient accumulation of 1-alkyl-2-lyso-GPC (lysoPAF). Raji PAF metabolism was temperature-dependent, with 96 ± 0.5% of 50 nM and 93 ± 0.8% of 1 nM [³H]PAF (mean ± S.E.M., n=2) remaining as a product migrating with PAF after 128 minute incubations at 4 °C. These experiments indicate that the metabolism of PAF in Raji B-lymphoblasts is similar to that in T-lymphocytes and MOLT-4 lymphoblasts, and this metabolism is negligible at 4 °C.

*The metabolism of CPAF in Raji cells* 1-alkyl-2-N-carbamyl-GPC (CPAF) is a recently described synthetic PAF analog that has PAF agonist qualities, but is not susceptible to PLA₂ or acetylhydrolase enzymatic degradation like native PAF [121]. Studies were undertaken to examine whether CPAF was metabolized by lymphocytes. As shown in Figure 11, when Raji or MOLT-4 lymphoblasts were incubated with 1 nM [³H]CPAF for times up to two hours and the products examined by TLC, a vast majority (> 95%) of the radiolabel was found in a product migrating with CPAF (synthesized as outlined in Appendix I). These
FIGURE 11. DISTRIBUTION OF LABEL IN PRODUCTS AFTER INCUBATION OF 1-[\textsuperscript{3}H]ALKYL-2-N-METHYLCARBAMYL-GPC WITH RAJI CELLS.

1-[\textsuperscript{3}H]alkyl-2-N-methylcarbamyl-GPC (1 nM) was incubated with Raji lymphoblasts for 128 min at 37 °C, and the lipids were extracted and separated as described in Figure 5.
experiments indicated that CPAF, a PAF agonist that has been shown not to be metabolized by neutrophils, was also not metabolized by lymphocytes.

D. PAF RADIOLIGAND BINDING STUDIES

The next series of experiments were designed to examine whether human lymphocytes expressed PAF receptors. Since cells that would be expected to be contaminants in populations of purified human lymphocytes (i.e., neutrophils, monocytes and platelets) all express PAF receptors, lymphocyte cell lines were utilized. Because ligand metabolism can give spurious binding results, PAF binding studies were conducted at 4 °C, conditions under which we have demonstrated minimal lymphocyte PAF metabolism. PAF binding studies were initiated using the method of O'Flaherty et al. [28], which was used to document PAF binding to human neutrophils.

*Human neutrophil PAF binding studies.* The first PAF radioligand binding experiments used human neutrophils, since they have previously been shown to express PAF receptors [26-28]. Binding studies conducted at 4 °C by incubating 5 x 10⁶ cells/ml with 200 pM of [³H]PAF, with or without 1 μM unlabeled PAF, demonstrated that human neutrophils specifically bound [³H]PAF. As shown in Figure 12, [³H]PAF specific binding reached saturation by 120 minutes. These experiments confirmed our methodology using neutrophils as a positive control.

Human polymorphonuclear leukocytes (PMNs) (5 x $10^6$ cells in 1 ml of HBSS) were incubated with 200 pM $[^3]$H]PAF for the indicated time and harvested as described in Methods. Specific binding was the difference between PAF binding in the absence and presence of excess unlabelled PAF (1 μM). Each point is the mean ± S.E.M. of the fraction of $[^3]$H]PAF specifically bound in 2 separate experiments utilizing triplicate samples.
FIGURE 13. KINETICS OF $[^3$H$]$PAF BINDING TO RAJI LYMPHOBLASTS AT 4°C.

Raji lymphoblasts ($5 \times 10^6$ cells in 1 ml of HBSS) were incubated with 200 pM $[^3$H$]$PAF for the indicated time and harvested as described in Figure 12. Each point is the mean ± S.E.M. of the fraction of $[^3$H$]$PAF specifically bound in 4-6 separate experiments utilizing triplicate samples.
Raji lymphoblast binding studies. Preliminary $[^3]$H]PAF binding studies indicated that while MOLT-4 lymphoblasts did not contain PAF binding sites, Raji lymphoblasts specifically bound $[^3]$H]PAF. The kinetics and reversibility of PAF binding were next examined in this Burkitt lymphoma-derived B cell line. Raji lymphoblasts ($5 \times 10^6$ cells/ml) were incubated in triplicate with $200 \text{ pM}$ of $[^3]$H]PAF with or without $1 \text{ nM}$ of unlabeled PAF, and the binding was quantified as described in Methods. As indicated in Figure 13, specific binding reached saturation by 80 min. Specific binding increased linearly between $2.5 - 10 \times 10^6$ cells/ml (Figure 14). The reversibility of PAF binding was examined by incubating Raji lymphoblasts under the above conditions for 120 min followed by addition of unlabeled PAF, with the binding quantified 20, 40, 60 and 120 min later. Addition of $1 \text{ nM}$ unlabeled PAF dissociated $41 \pm 9\%$ (mean $\pm$ S.E.M., $n = 4$) of specifically-bound $[^3]$H]PAF from the Raji lymphoblasts after 120 min. Addition of $5 \text{ nM}$ unlabeled PAF did not result in a greater amount of dissociated $[^3]$H]PAF (results not shown). These studies indicated that specific PAF binding to Raji lymphoblasts was demonstrable at $4 \degree \text{C}$ with saturation occurring by 80 min. This binding was only partially reversible.

Scatchard analysis to determine the binding affinity and receptor number. The affinity and number of PAF binding sites in Raji lymphoblasts were determined by constructing saturation binding isotherms. As demonstrated in Figure 15, PAF specific binding increased with concentration of labeled ligand and reached
FIGURE 14. $[^3H]PAF$ BINDING VS RAJI LYMPHOBLAST CELL NUMBER.

Various concentrations of Raji lymphoblasts (1.25 - $10 \times 10^6$ cells in 1 ml of HBSS) were incubated with 200 pM $[^3H]PAF$ for 120 minutes and harvested as described in Figure 12. Each point is the mean of $[^3H]PAF$ (dpms) specifically bound in a single experiment utilizing triplicate samples.
FIGURE 15. SATURATION KINETICS AND SCATCHARD ANALYSIS OF 
$[^3$H]$PAF$ BINDING TO RAJI LYMPHOBLASTS.

Saturation binding isotherms were constructed (inset) by equilibrium (120 min) 
binding of 0.02 - 8.5 nM $[^3$H]$PAF$ to $5 \times 10^6$ cells. The data were subjected to 
Scatchard analysis. The data pictured are from a single experiment using triplicate 
samples and is representative of 9 separate experiments.
saturation. The data were then subjected to Scatchard analysis [109] to quantitate the number of binding sites present and their apparent affinity. Scatchard analysis of 9 separate experiments gave a linear plot (Figure 15), indicating a single population of PAF binding sites. The equilibrium dissociation constant ($K_D$), representing the affinity of the PAF binding site, was $2.3 \pm 0.3 \text{nM}$. The maximal binding ($B_{max}$), representing the total number of specific binding sites present, was $148 \pm 36 \text{fmoles/5 x 10}^6 \text{cells}$. Assuming an equimolar ligand-receptor complex, the above $B_{max}$ corresponds to $17,800 \pm 3600$ binding sites per Raji lymphoblast.

Although the rate of PAF binding was determined using 200 pM [$^3\text{H}$]PAF, the saturation binding isotherms subjected to Scatchard analysis used [$^3\text{H}$]PAF concentrations as low as 20 pM. To address the potential problem of the binding of lower [$^3\text{H}$]PAF concentrations not reaching saturation within 120 min, additional binding experiments with 20 pM [$^3\text{H}$]PAF at 120 min and 180 min incubation periods were conducted. As shown in Table 2, two separate experiments indicated no difference in specific binding between the two times. Thus, 120 min was an appropriate incubation time for conducting saturation binding isotherms in this system.

Saturation binding isotherms did not use [$^3\text{H}$]PAF concentrations $>10 \text{nM}$ because the concentrations of unlabeled PAF necessary to displace these higher concentrations of [$^3\text{H}$]PAF are not achievable due to the limited solubility of PAF
in aqueous solutions. To assess Raji lymphoblasts for the presence of a second lower affinity binding site, data from competition binding studies with 1 nM \( [^3H]PAF \) incubated with unlabeled PAF (10 pM - 1 \( \mu \)M) (Figure 16) were subjected to Scatchard analysis. Two distinct types of binding sites were found. A single high affinity site with binding constants (\( K_D = 5.7 \pm 1.4 \) nM, \( B_{\text{max}} = 280 \pm 50 \) fmoles/5 x 10^6 cells, \( n = 5 \)) similar to those found by analysis of saturation binding isotherms was identified. The second site was of substantially lower affinity (\( K_D > 1 \) \( \mu \)M) with nearly infinite binding capacity (\( B_{\text{max}} > 100 \) pmoles/5 x 10^6 cells).

**Competition binding studies with PAF receptor antagonists.** To demonstrate the specificity of Raji lymphoblast PAF binding, competition binding studies with lysoPAF, CPAF and PAF receptor antagonists were conducted. As shown in Figure 17 and Figure 18, both PAF receptor antagonists and CPAF competed with \( [^3H]PAF \) for binding to Raji lymphoblasts. The physiologically inactive [5] precursor/metabolite lysoPAF did not compete with PAF for binding. The PAF receptor antagonists exhibited the following order of potency: Ro 19-3704 = CV-6209 > CV-3988 > BN52021 > Alprazolam. As shown in Table 3, the concentrations needed to compete with 50 % of PAF binding (IC\text{50}) ranged from 2 nM to 2 \( \mu \)M. CV-6209 and Ro19-3704 are approximately as potent as native PAF in competing with \( [^3H]PAF \) for binding to Raji cells.

*The effect of calcium channel antagonists on \([^3H]PAF\) binding.* Studies by
**TABLE 2. SPECIFIC BINDING OF 20 pM [³H]PAF TO RAJI LYMPHOBLASTS AT 120 AND 180 MINUTES.**

Raji lymphoblasts were incubated with 20 pM [³H]PAF and 1 µM unlabeled PAF or BSA alone for 120 or 180 minutes, and the binding quantitated as described in *Methods*. Each value is the mean ± S.D. of dpms bound to cells in the presence of 1 µM PAF or BSA alone.

<table>
<thead>
<tr>
<th>BSA</th>
<th>1 µM PAF</th>
<th>Specific Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>447 ± 58</td>
<td>246 ± 27</td>
</tr>
<tr>
<td>180 min</td>
<td>448 ± 53</td>
<td>261 ± 7</td>
</tr>
<tr>
<td>Exp. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 min</td>
<td>499 ± 26</td>
<td>275 ± 10</td>
</tr>
<tr>
<td>180 min</td>
<td>488 ± 33</td>
<td>323 ± 19</td>
</tr>
</tbody>
</table>
FIGURE 16. COMPETITION OF $[^3H]PAF$ BINDING TO RAJI LYMPHOBLASTS BY PAF.

The $5 \times 10^6$ cells in 1 ml of HBSS were incubated with 1 nM $[^3H]PAF$ and different concentrations of unlabeled PAF, or BSA alone for 120 min and harvested as described in Figure 12. The data are expressed as the percentage of specific binding observed at each concentration of unlabeled PAF. Each point is the mean ± S.E.M. of triplicate determinations from 5 separate experiments.
FIGURE 17. COMPETITION OF [3H]PAF BINDING TO RAJI LYMPHOBLASTS BY PAF RECEPTOR ANTAGONISTS.

Raji lymphoblasts were incubated with 0.5 nM [3H]PAF and different concentrations of the PAF receptor antagonists CV-6209 (○), alprazolam (■), lysoPAF (▲), or BSA alone as described in Figure 16. The data are expressed as the percentage of specific binding observed at each concentration of unlabeled compound. Each point is the mean ± S.E.M. of triplicate determinations from at least 3 separate experiments.
FIGURE 18. COMPETITION OF [³H]PAF BINDING TO RAJI LYMPHOBLASTS BY CPAF.

Raji lymphoblasts were incubated with [³H]PAF and different concentrations of CPAF or BSA alone as described in Figure 17. The data are expressed as the percentage of specific binding observed at each concentration of CPAF. Each point is the mean ± S.E.M. of triplicate determinations from at least 4 separate experiments.
### TABLE 3. INHIBITION OF [³H]PAF SPECIFIC BINDING TO RAJI LYMPHOBLASTS BY PAF, CPAF AND PAF RECEPTOR ANTAGONISTS.

Raji lymphoblasts were incubated with [³H]PAF and different concentrations of PAF, CPAF or PAF receptor antagonists as outlined in Figure 17 and Figure 18. The resultant curves were constructed by computer. Each value is the mean ± S.E.M. IC₅₀ with \( n = 3-4 \).

<table>
<thead>
<tr>
<th>Binding Inhibitor</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAF</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>CPAF</td>
<td>7.6 ± 0.9</td>
</tr>
<tr>
<td>Ro19-3704</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>CV-6209</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>CV-3988</td>
<td>178 ± 53</td>
</tr>
<tr>
<td>BN52021</td>
<td>470 ± 130</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>2300 ± 300</td>
</tr>
</tbody>
</table>
FIGURE 19. COMPETITION OF $[^3]$H]PAF BINDING TO RAJI LYMPHOBLASTS BY CALCIUM CHANNEL ANTAGONISTS.

Raji lymphoblasts were incubated with $[^3]$H]PAF and different concentrations of the voltage-sensitive calcium channel antagonists diltiazem ( ■ ), verapamil ( ● ), nimodipine ( ▲ ), or BSA alone as described in Figure 17. The data are expressed as the percentage of specific binding observed at each concentration of calcium channel antagonist. Each point is the mean ± S.E.M. of triplicate determinations from at least 4 separate experiments.
**TABLE 4. INHIBITION OF [³H]PAF SPECIFIC BINDING TO RAJI LYMPHOBLASTS BY CALCIUM CHANNEL ANTAGONISTS.**

Raji lymphoblasts were incubated with [³H]PAF and different concentrations of the calcium channel antagonists as outlined in Figure 19. The resultant curves were constructed by computer. Each value is the mean ± S.E.M. IC₅₀ with n = 4.

<table>
<thead>
<tr>
<th>Calcium Channel Antagonist</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>4.8 ± 1.3</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>9.3 ± 2.8</td>
</tr>
</tbody>
</table>
Valone [53,54] and others [122,123] indicated that the calcium channel antagonists verapamil and diltiazam inhibit PAF-induced platelet aggregation at doses which are in the range of the IC₅₀s for [³H]PAF binding. To assess whether calcium channel antagonists were active at the Raji lymphoblast PAF receptor, verapamil, diltiazam and nimodipine were tested for the ability to compete with [³H]PAF for binding to Raji cells. As shown in Figure 19 and Table 4, these compounds competed with [³H]PAF for binding to Raji cells in a dose-dependent manner. The IC₅₀s of these compounds were similar, ranging from 3 - 9 μM.

These studies indicate that the Raji PAF receptor is similar to the platelet PAF receptor with regard to the ability of verapamil and diltiazem to compete with PAF for binding. However, the finding that nimodipine, a much more potent calcium channel antagonist [56], is not more potent in competing with PAF, suggests that these compounds are not exerting this effect by acting as voltage-sensitive calcium channel antagonists.

*Binding of CPAF to Raji lymphoblasts.* Competition binding studies with CPAF indicated that this analog is only slightly less potent than native PAF. Since a non-hydrolyzable PAF agonist would be valuable in biological studies, especially with cells such as lymphocytes that readily degrade PAF, binding studies with [³H]CPAF were conducted to assess its binding characteristics. The kinetics of [³H]CPAF binding at 4 °C were similar to those found for PAF, with saturation occurring by 120 minutes (Figure 20). The affinity and number of CPAF binding
sites in Raji lymphoblasts were determined by constructing saturation binding isotherms. As shown in Figure 21, the specific binding of CPAF increased with concentration of radiolabeled ligand and reached saturation. The data was then subjected to Scatchard analysis to quantitate the number of CPAF binding sites and their apparent affinity. Scatchard analysis of 4 separate experiments (Figure 21) gave a linear plot, indicating a single population of CPAF binding sites. The equilibrium dissociation constant ($K_D$), representing the affinity of the CPAF binding site, was $2.7 \pm 0.6$ nM. The maximal binding ($B_{\text{max}}$), representing the total number of specific binding sites present, was 123 fmoles/5 x $10^6$ cells. This $B_{\text{max}}$ is very close to the value obtained using $[^3H]PAF$, indicating that these two compounds probably share the same binding site. These experiments indicated that CPAF binds the PAF receptor with binding characteristics similar to PAF.

E. THE EFFECT OF PAF ON RAJI INTRACELLULAR CALCIUM LEVELS

*PAF-induced intracellular calcium mobilization.* To determine whether PAF specific binding sites on Raji lymphoblasts were functionally active, changes in intracellular free calcium ([Ca$^{2+}$]) induced by PAF were measured using the fluorescent probe fura-2. Resting [Ca$^{2+}$] in unstimulated cells was 148 ± 7 nM (mean ± S.E.M., $n = 40$) at 37 °C. PAF caused a transient increase in intracellular free calcium in a dosage-dependent manner. As illustrated in Figure 22, [Ca$^{2+}$] began to increase 5 - 10 seconds after the addition of PAF and returned to baseline within 2 minutes. While the magnitude of the change in [Ca$^{2+}$],
Raji lymphoblasts (5 x 10⁶ cells in 1 ml of HBSS) were incubated with 1 nM [³H]CPAF for the indicated time and harvested as described in Figure 12. Each point is the mean ± S.E.M. of the fraction of [³H]CPAF specifically bound in 2 separate experiments utilizing triplicate samples.
FIGURE 21. SATURATION KINETICS AND SCATCHARD ANALYSIS OF $^{3}$HCPAF BINDING TO RAJI LYMPHOBLASTS.

Saturation binding isotherms were constructed (inset) by equilibrium (120 min) binding of 0.1 - 16 nM $^{3}$HCPAF to 5 x 10$^{6}$ cells. The data were subjected to Scatchard analysis. The data pictured are the mean values from 4 separate experiments utilizing triplicate samples.
increased with increasing doses of PAF, the time to achieve maximal response decreased. Repeated stimulation with PAF resulted in little change in baseline 
$[\text{Ca}^{2+}]_i$ (results not shown). A dose-response curve characterizing the relationship between maximal changes in $[\text{Ca}^{2+}]_i$ and PAF concentration is shown in Figure 23. The change in $[\text{Ca}^{2+}]_i$ was evident at a PAF concentration as low as 10 pM and was maximal at 100 nM. The EC$_{50}$ of the curve is 6.3 nM, which is similar to the K$_D$ calculated from the binding studies. PAF did not affect cellular integrity at any concentration used as assessed by trypan blue dye exclusion. Because Raji lymphoblasts quickly metabolize PAF to lysoPAF at 37 °C, lysoPAF was tested for the ability to increase $[\text{Ca}^{2+}]_i$. LysoPAF (1 µM) did not increase $[\text{Ca}^{2+}]_i$ above resting values (results not shown), confirming that the effect of PAF was not mediated by this metabolite.

Effect of Ca$^{2+}$-free media on PAF-induced intracellular calcium mobilization. Studies with platelets [47-49], neutrophils [49], monocytes [41,50] and PC-12 cells [51,52] indicate that the PAF-induced intracellular calcium mobilization is due to both extracellular Ca$^{2+}$ influx and release of intracellular stores. To assess the origin of the increase in $[\text{Ca}^{2+}]_i$, fura-2 fluorescence studies were conducted in HBSS without extracellular calcium plus 100 µM EGTA. Under these essentially Ca$^{2+}$-free ($[\text{Ca}^{2+}] < 10$ nM) conditions, addition of PAF did not result in a measurable increase in $[\text{Ca}^{2+}]_i$ above control values (results not shown). This suggests that the vast majority of Raji PAF-induced intracellular Ca$^{2+}$ mobilization
FIGURE 22. TYPICAL EXAMPLES OF PAF-INDUCED INTRACELLULAR CALCIUM MOBILIZATION IN RAJI LYMPHOBLASTS.

Fura-2-loaded Raji lymphoblasts were incubated with HBSS containing 1.4 mM CaCl$_2$ and challenged with 1 to 100 nM PAF (arrow). The magnitude as well as the rate of [Ca$^{2+}$]$_i$ increase are proportional to the amount of PAF available ((a) 100 nM, (b) 10 nM, and (c) 1 nM PAF).
FIGURE 23. CONCENTRATION DEPENDENCE OF PAF-INDUCED INTRACELLULAR CALCIUM MOBILIZATION IN RAJI LYMPHOBLASTS.

Fura-2-loaded Raji lymphoblasts were treated with 10 pM - 1 μM PAF as described in Figure 22. The peak change in [Ca^{2+}]_i was calculated. The curve was constructed by computer and had an EC_{50} of 6.3 nM. The values are mean ± S.E.M. of 3 - 6 separate experiments.
is due to extracellular Ca\(^{2+}\) influx.

*Effect of PAF receptor antagonists on calcium flux.* To demonstrate the specificity of PAF-induced \([\text{Ca}\^{2+}]_i\) changes, the ability of PAF receptor antagonists to inhibit PAF-induced calcium mobilization was tested. As shown in Figure 24, a large increase in \([\text{Ca}\^{2+}]_i\) was evident within 20 seconds after the addition of 1 nM PAF. When the cells were pretreated with 1 \(\mu\)M CV-3988, however, subsequent addition of 1 nM PAF resulted in little change in \([\text{Ca}\^{2+}]_i\). BN52021 (100 \(\mu\)M) also inhibited the PAF-induced changes in \([\text{Ca}\^{2+}]_i\) (*results not shown*). To further demonstrate that the PAF-induced calcium flux was due to PAF interacting with its receptor, the ability of CV-6209 and alprazolam to inhibit PAF-induced increases in \([\text{Ca}\^{2+}]_i\) were tested. As shown in Figure 25, these PAF receptor antagonists attenuated 10 nM PAF-induced calcium mobilization in a dose-dependent manner. A comparison between the IC\(_{50}\)s of the antagonists for PAF-induced calcium mobilization and competition binding assays (Table 5) strongly suggests that these compounds inhibit PAF-induced calcium mobilization by competing with PAF for binding to its receptor. Treatment of Raji cells with the PAF receptor antagonists CV-6209, CV-3988, BN52021 and alprazolam did not result in an increase in \([\text{Ca}\^{2+}]_i\), indicating that these compounds do not have measurable PAF agonistic effects on the Raji lymphoblast PAF receptor. None of these compounds at the concentrations used affected the viability of the Raji lymphoblasts in either the binding or calcium studies as measured by trypan blue
FIGURE 24. INHIBITION OF PAF-INDUCED INTRACELLULAR CALCIUM MOBILIZATION IN RAJI LYMPHOBLASTS BY PAF RECEPTOR ANTAGONISTS.

Raji lymphoblasts were treated with 1 nM PAF (arrow) in the absence (a) and the presence (b) of 1 μM CV-3988. Pretreatment of the cells with CV-3988 for 30 sec abolished the PAF-dependent Ca^{2+} mobilization. The fura-2 fluorescence tracings represent 4 separate experiments. Treatment of the cells with 100 μM BN52021 resulted in similar inhibition.
Fura-2-loaded Raji lymphoblasts were pretreated with various doses of CV-6209 (●), alprazolam (■) or lysoPAF (▲) for 30 seconds, followed by 10 nM PAF. The peak change in [Ca^{2+}]_i was calculated. Each point is the mean ± S.E.M. of the percentage of peak change of [Ca^{2+}]_i induced by PAF alone with 3-4 separate experiments utilizing duplicate samples.
TABLE 5. COMPARISON BETWEEN THE INHIBITION OF PAF BINDING AND PAF-INDUCED INTRACELLULAR CALCIUM MOBILIZATION IN RAJI CELLS BY PAF ANTAGONISTS CV-6209 AND ALPRAZOLAM.

The concentrations of the PAF receptor antagonists CV-6209 and alprazolam to compete with 50% of [\(^3\)H]PAF binding (Figure 17) and inhibit 50% of 10 nM PAF-induced intracellular calcium mobilization (Figure 25) are expressed as mean ± S.E.M. \( IC_{50} \)s for comparison.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( IC_{50} ) Binding (nM)</th>
<th>( IC_{50} ) Calcium (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV-6209</td>
<td>2.9 ± 0.5</td>
<td>3.5 ± 1.6</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>2,300 ± 300</td>
<td>7,200 ± 3000</td>
</tr>
</tbody>
</table>
FIGURE 26. CONCENTRATION DEPENDENCE OF CPAF-INDUCED INTRACELLULAR CALCIUM MOBILIZATION IN RAJI LYMPHOBLASTS.

Fura-2-loaded Raji lymphoblasts were treated with 100 pM - 1 μM CPAF as described in Figure 22. The peak change in [Ca^{2+}]_i was calculated. The curve was constructed by computer and had an EC_{50} of 3.0 nM. The values are mean ± S.E.M. of 4 - 6 separate experiments.
exclusion.

*The effect of CPAF on calcium flux.* Although binding studies indicated that CPAF binds to the Raji lymphoblast PAF receptor, fura-2 fluorescent studies were necessary to determine whether this non-hydrolyzable PAF analog was a PAF agonist. As shown in Figure 26, treatment of Raji lymphoblasts with 100 pM - 1 µM CPAF resulted in an increase in [Ca\(^{2+}\)]\(_i\) in a dose-dependent manner. The EC\(_{50}\) of the resultant curve is 3.0 nM, which is similar to the K\(_D\) calculated from the \[^3\text{H}]\text{CPAF}\) binding studies. The results of these experiments indicate that CPAF is a more metabolically stable PAF receptor agonist.

**F. THE EFFECT OF PAF ON RAJI PROLIFERATION**

The demonstration of functional PAF receptors in Raji lymphoblasts prompted an examination of the effects of PAF agonists and antagonists on Raji function. The functional parameter examined in these tumor cells was the ability of the PAF receptor to modulate proliferation (as assessed by measurement of \[^3\text{H}]\text{TdR}\) incorporation) in these cells. To these ends, both spontaneous proliferation and proliferation in the presence of cytokines and other agents were examined.

*The effect of PAF and CPAF on Raji spontaneous proliferation.* The ability of PAF to modulate Raji spontaneous proliferation was examined. Treatment of Raji lymphoblast cultures with 10 pM - 10 µM PAF for 24 hours did not affect \[^3\text{H}]\text{TdR}\) uptake as shown in Figure 27. Since PAF is readily metabolized by these
cells, the more stable PAF agonist CPAF was used in the further exploration of the effects of PAF receptor activation on the proliferative process in this cell line. Cells were cultured for 24 or 48 hours with picomolar to low micromolar concentrations of CPAF, either with 5% FBS or without serum. As demonstrated in Figure 28, CPAF did not affect Raji spontaneous proliferation (at non-toxic doses) under these conditions.

The effect of BN52021 on Raji spontaneous proliferation. A possible modulatory effect of PAF receptor blockade on Raji proliferation was assessed by using the PAF receptor antagonist BN52021. As shown in Figure 29, treatment with doses of BN52021 > 100 μM resulted in a decrease in [³H]TdR incorporation into spontaneously proliferating Raji lymphoblasts. To test whether this antimitotic effect was due to the interaction of BN52021 with a PAF receptor, the ability on CPAF to reverse BN52021's effect was examined. As shown in Figure 30, CPAF, at doses up to 100 nM, did not appreciably affect the antimitotic effect of 250 μM BN52021. This finding suggests that blockade of the PAF receptor was probably not responsible for the anti-proliferative effect of high doses of BN52021. The results of these experiments fail to provide evidence that the PAF receptor expressed on Raji lymphoblasts is involved in the spontaneous proliferative process in these tumor cells.

The effect of CPAF on Raji proliferation in the presence of cytokines. The cytokines interleukin-1 (IL-1), interleukin-2 (IL-2) and interferon-gamma (γ-IFN)
have been shown to have effects on B cells by interaction with specific receptors [124-129]. The ability of these cytokines to modulate Raji spontaneous proliferation was examined by treating Raji cells with 0.1 - 100 U/ml of IL-1α, IL-2 and γ-IFN for 24 and 48 hours, both in the presence and absence of serum. As shown in Figures 31,32 and 33, these cytokines at the indicated concentrations did not affect Raji proliferation as assessed by [³H]TdR incorporation. Addition of 1 - 100 nM CPAF to 100 U/ml of IL-1α, IL-2 or γ-IFN also did not change Raji proliferation above unstimulated levels (results not shown).

The effect of CPAF on Raji proliferation in the presence of LPS and fibrinogen. Bacterial lipopolysaccharide (LPS) has been shown to be a potent B cell mitogen [130]. The ability of LPS treatment to affect Raji lymphoblast proliferation was next assessed. As shown in Figure 34, 0.1 - 100 μg/ml LPS did not modulate Raji proliferation under the experimental conditions outlined above. Addition of 1 - 100 nM of CPAF to 100 μg/ml of LPS also did not change Raji proliferation above unstimulated levels (results not shown). In contrast to previously reported findings [131], treatment with fibrinogen (0.1 -100 μg/ml) did not stimulate Raji proliferation (Figure 35). Addition of 1-100 nM CPAF to 100 μg/ml fibrinogen also had no appreciable affect on Raji proliferation (results not shown).

The effect of CPAF on Raji proliferation stimulated by sfBT conditioned media. Recent studies in the laboratory of Shaw [111] indicate that Epstein-Barr virus (EBV)-transformed B cell lines can be cultured in the absence of serum. These
FIGURE 27. THE EFFECT OF PAF ON RAJI LYMPHOBLAST SPONTANEOUS PROLIFERATION.

Various concentrations of PAF were added to cultures containing $1 \times 10^4$ Raji lymphoblasts in complete media containing 10% CS. The resultant spontaneous proliferation was detected after a 24 hour incubation and an 8 hour terminal pulse with $[\text{H}]\text{TdR}$ as described in Methods. Each value represents the mean ± S.E.M. of triplicate cultures from one representative experiment of four conducted.
FIGURE 28. THE EFFECT OF CPAF ON RAJI LYMPHOBLAST SPONTANEOUS PROLIFERATION.

Various concentrations of the PAF agonist CPAF were added to cultures containing $1 \times 10^4$ Raji lymphoblasts. The resultant spontaneous proliferation was detected by an 8 hour terminal pulse with $[^3]$H]Tdr, following incubation in complete media containing 10% CS for 24 (■) or 48 (▲) hours, or incubation in complete media without serum for 24 (▲) or 48 (▲) hours. Each value represents the mean ± S.E.M. of triplicate cultures from one representative experiment of two conducted.
FIGURE 29. THE EFFECT OF BN52021 ON RAJI LYMPHOBLAST SPONTANEOUS PROLIFERATION.

Various concentrations of the PAF receptor antagonist BN52021 were added to cultures containing $1 \times 10^4$ Raji lymphoblasts. The resultant spontaneous proliferation was detected by an 8 hour terminal pulse with $[^3]$H]TdR, following incubation in complete media containing 10% CS for 24 (■) or 48 (▲) hours, or incubation in complete media without serum for 24 (□) or 48 (▲) hours. Each value represents the mean ± S.E.M. of triplicate cultures from one representative experiment of two conducted.
FIGURE 30. CPAF DOES NOT REVERSE THE INHIBITORY EFFECTS OF BN52021 ON RAJI LYMPHOBLAST SPONTANEOUS PROLIFERATION.

Raji lymphoblasts (1 x 10⁴) were incubated with either DMSO (control), 250 μM BN52021, or 250 μM BN52021 with various concentrations of CPAF. The resultant spontaneous proliferation was detected by an 8 hour terminal pulse with [³H]TdR, following incubation in complete media containing 10% CS for 24 (■) or 48 (▲) hours, or incubation in complete media without serum for 24 (□) or 48 (△) hours. Each value represents the mean ± S.E.M. of triplicate cultures from one experiment.
FIGURE 31. THE EFFECT OF IL-1 ON RAJI LYMPHOBLAST SPONTANEOUS PROLIFERATION.

Various concentrations of interleukin-1 (IL-1) were added to cultures containing $1 \times 10^4$ Raji lymphoblasts. The resultant spontaneous proliferation was detected by an 8 hour terminal pulse with $[^3H]$TdR, following incubation in complete media containing 10% CS for 24 (■) or 48 (▲) hours, or incubation in complete media without serum for 24 (□) or 48 (▲) hours. Each value represents the mean ± S.E.M. of triplicate cultures from one representative experiment of two conducted.
FIGURE 32. THE EFFECT OF IL-2 ON RAJI LYMPHOBLAST SPONTANEOUS PROLIFERATION.

Various concentrations of interleukin-2 (IL-2) were added to cultures containing $1 \times 10^4$ Raji lymphoblasts. The resultant spontaneous proliferation was detected by an 8 hour terminal pulse with $[^3]H$TdR, following incubation in complete media containing 10% CS for 24 (■) or 48 (▲) hours, or incubation in complete media without serum for 24 (□) or 48 (△) hours. Each value represents the mean ± S.E.M. of triplicate cultures from one representative experiment of two conducted.
FIGURE 33. THE EFFECT OF GAMMA INTERFERON ON RAJI LYMPHOBLAST SPONTANEOUS PROLIFERATION.

Various concentrations of immune (gamma) interferon (γ-IFN) were added to cultures containing 1 x 10^4 Raji lymphoblasts. The resultant spontaneous proliferation was detected by an 8 hour terminal pulse with [3H]TdR, following incubation in complete media containing 10% CS for 24 (■) or 48 (▲) hours, or incubation in complete media without serum for 24 (□) or 48 (▲) hours. Each value represents the mean ± S.E.M. of triplicate cultures from one representative experiment.
FIGURE 34. THE EFFECT OF LPS ON RAJI LYMPHOBLAST SPONTANEOUS PROLIFERATION.

Various concentrations of bacterial lipopolysaccharide (LPS) were added to cultures containing $1 \times 10^4$ Raji lymphoblasts. The resultant spontaneous proliferation was detected by an 8 hour terminal pulse with $[^3]$H]Tdr, following incubation in complete media containing 10% CS for 24 (■) or 48 (▲) hours, or incubation in complete media without serum for 24 (□) or 48 (▲) hours. Each value represents the mean ± S.E.M. of triplicate cultures from one representative experiment of two conducted.
FIGURE 35. THE EFFECT OF FIBRINOGEN ON RAJI LYMPHOBLAST SPONTANEOUS PROLIFERATION.

Various concentrations of human fibrinogen were added to cultures containing $1 \times 10^4$ Raji lymphoblasts. The resultant spontaneous proliferation was detected by an 8 hour terminal pulse with $[^3]$H]TdR, following incubation in complete media containing 10% CS for 24 (□) or 48 (▲) hours, or incubation in complete media without serum for 24 (△) or 48 (▲) hours. Each value represents the mean ± S.E.M. of triplicate cultures from one representative experiment of two conducted.
FIGURE 36. THE EFFECT OF CONDITIONED MEDIA ON RAJI LYMPHOBLAST SPONTANEOUS PROLIFERATION.

Various concentrations of conditioned media from sfBT lymphoblasts were added to cultures containing $1 \times 10^4$ Raji lymphoblasts. The resultant spontaneous proliferation was detected by an 8 hour terminal pulse with $[^3]$H]TdR, following incubation in complete media without serum for 24 (□) or 48 (△) hours. Each value represents the mean ± S.E.M. of triplicate cultures from one representative experiment of three conducted.
cell lines are thought to secrete growth factors that act upon the cells in an autocrine manner. The spent media from one of these serum-free cell lines, sfBT, has been shown to support growth of other cell lines [111]. The ability of spent media from sfBT to modulate Raji proliferation was assessed by treating Raji cultures with sfBT conditioned media provided by Dr. Shaw. As shown in Figure 36, sfBT conditioned media increased Raji proliferation as measured by \(^3\)H-TdR incorporation. CPAF (1-100 nM) treatment did not result in a detectable change in the trophic effects of sfBT conditioned media (results not shown). These preliminary studies indicated that Raji spontaneous proliferation was increased by sfBT conditioned media, but was unaffected by treatment with IL-1, IL-2, -IFN, fibrinogen and LPS as outlined above. Treatment of Raji cultures with CPAF, alone, or with the above agents, did not affect Raji proliferation as assessed by \(^3\)H-TdR incorporation studies.

G. IDENTIFICATION OF FUNCTIONAL PAF RECEPTORS ON OTHER B CELL LINES

The finding that Raji, an Epstein-Barr Virus (EBV)-immortalized B cell line from a Burkitt lymphoma [120] contains functional PAF receptors, prompted the examination of other established B and T cell lines for the presence of PAF receptors. Twelve other lymphoid cell lines were surveyed for the presence of PAF receptors by binding studies and testing whether PAF treatment resulted in intracellular calcium mobilization.
**Description of cell lines tested for PAF receptors.** As shown in Table 6, both B and T cell lines, with and without the EBV genome were utilized. Raji [120], Daudi [132] and P3HR-1 [133] are EBV DNA-positive cell lines derived from Burkitt lymphomas. Human umbilical cord blood (CB-1) and peripheral blood (PB-1) B cell lines were established by Dr. James Shaw by infection in vitro using virus strain B95-8 [134]. Dakiki [135] is an IgA-secreting cell line that was derived by EBV infection of B cells from a patient with nasopharyngeal carcinoma. B95-8 [134] is a tamarin (Saguinus oedipus) peripheral blood B cell line from a throat washing of a patient with infectious mononucleosis. sfBT is a serum-free cell line derived from B95-8 cells [111]. BJAB was derived from a non-Burkitt B cell lymphoma and is without EBV DNA [136,137]. BJA/HR-1 was established by EBV infection of BJAB [138]. Also used in these studies were two acute lymphoblastic leukemia T cell lines (MOLT-4, CCRF/CEM) [114,139] and the non-B, non-T cell line NALM-6 [140].

**Binding of [3H]PAF to the cell lines.** The above lymphoid cell lines were tested for the ability to specifically bind [3H]PAF. As shown in Table 7, six of the thirteen cell lines specifically bound [3H]PAF. The amount of [3H]PAF that was specifically bound differed considerably among the cell lines. This binding was specific for PAF, as 1 μM of the inactive precursor/metabolite lysoPAF did not compete with [3H]PAF for binding to these cell lines (*data not shown*). Based on these studies, lymphoblastoid cell lines other than Raji contain specific PAF
binding sites.

*Testing the cell lines for PAF-induced calcium mobilization.* To test if the binding sites found on the lymphoid cell lines were functional PAF receptors, the cells were examined for PAF-induced intracellular calcium mobilization using the fluorescent probe fura-2. Of all the cell lines listed in Table 6, four exhibited this phenomenon. In these responsive cells, $[\text{Ca}^{2+}]_i$ began to increase 5-10 seconds after addition of PAF and returned to baseline values within several minutes. As demonstrated in Table 7, peak changes in $[\text{Ca}^{2+}]_i$ induced by 1 $\mu$M PAF ranged from 34 nM to 144 nM. PAF treatment did not increase $[\text{Ca}^{2+}]_i$ above control values in cell lines that did not express PAF binding. Based on these studies, several other B lymphoid cell lines in addition to Raji contain functional PAF receptors.
### TABLE 6. CHARACTERISTICS AND ORIGIN OF LYMPHOID CELL LINES.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin (lymphoid origin)</th>
<th>EBV DNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Serum&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raji</td>
<td>Burkitt lymphoma (B)</td>
<td>+</td>
<td>10%</td>
</tr>
<tr>
<td>Daudi</td>
<td>Burkitt lymphoma (B)</td>
<td>+</td>
<td>10%</td>
</tr>
<tr>
<td>P3HR-1</td>
<td>Burkitt lymphoma (B)</td>
<td>+</td>
<td>5%</td>
</tr>
<tr>
<td>PBL-1</td>
<td>Peripheral blood (B)</td>
<td>+</td>
<td>10%</td>
</tr>
<tr>
<td>CB-1</td>
<td>Umbilical cord blood (B)</td>
<td>+</td>
<td>10%</td>
</tr>
<tr>
<td>BJAB</td>
<td>Non-Burkitt lymphoma (B)</td>
<td>-</td>
<td>15%</td>
</tr>
<tr>
<td>BJA/HR-1</td>
<td>EBV-converted BJAB (B)</td>
<td>-</td>
<td>10%</td>
</tr>
<tr>
<td>Dakiki</td>
<td>EBV-converted nasopharyngeal carcinoma (B)</td>
<td>+</td>
<td>10%</td>
</tr>
<tr>
<td>NALM-6</td>
<td>Acute lymphoblastic leukemia (non B, non T)</td>
<td>-</td>
<td>5%</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>Acute lymphoblastic leukemia (T)</td>
<td>-</td>
<td>10%</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>Acute lymphoblastic leukemia (T)</td>
<td>-</td>
<td>10%</td>
</tr>
<tr>
<td>B95-8</td>
<td>Peripheral Blood&lt;sup&gt;c&lt;/sup&gt; (B)</td>
<td>+</td>
<td>5%</td>
</tr>
<tr>
<td>sfBT</td>
<td>Peripheral Blood&lt;sup&gt;c&lt;/sup&gt; (B)</td>
<td>+</td>
<td>0%</td>
</tr>
</tbody>
</table>

<sup>a</sup>EBV DNA = Epstein-Barr virus DNA; + present, - absent

<sup>b</sup>Serum = Amount of FBS or CS (Raji) used to culture cells

<sup>c</sup>B95-8 and sfBT are from tamarin (*Saguinus oedipus*)
## TABLE 7. PAF SPECIFIC BINDING AND INTRACELLULAR CALCIUM MOBILIZATION IN LYMPHOID CELL LINES.

The cell lines described in Table 6 were incubated with 1 nM [³H]PAF ± 1 μM unlabeled PAF, and the binding quantitated as described in Methods. The amount of specific binding is expressed as mean ± S.E.M. of the percentage of total radiolabel specifically bound of at least four separate experiments using triplicate samples. The increase in baseline [Ca²⁺], induced by treatment of cells with 1 μM PAF was assessed as described in Methods and is expressed as mean ± S.E.M. of at least three separate experiments utilizing duplicate samples.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>% [³H]PAF Specifically Bound</th>
<th>Change in [Ca²⁺], nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raji</td>
<td>4.3 ± 0.5</td>
<td>144 ± 16</td>
</tr>
<tr>
<td>Daudi</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P3HR-1</td>
<td>1.0 ± 0.3</td>
<td>40 ± 11</td>
</tr>
<tr>
<td>PBL-1</td>
<td>0.8 ± 0.2</td>
<td>117 ± 21</td>
</tr>
<tr>
<td>CB-1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BJAB</td>
<td>1.5 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>BJA/HR-1</td>
<td>0.6 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Dakiki</td>
<td>1.9 ± 0.1</td>
<td>34 ± 9</td>
</tr>
<tr>
<td>NALM-6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B95-8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sfBT</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
A. THE USE OF TUMOR CELL LINES TO STUDY NORMAL LYMPHOCYTES

Many of the advances in modern immunology directly or indirectly involve the use of tumor cell lines. Tumor cell lines can serve as model systems or as sources of regulatory products such as cytokines. Examples of cell lines used as model systems to test cytokines include the B cell line CESS, which responds to B cell differentiation factors (BCDF) by differentiation and immunoglobulin secretion [141], and the B cell line WEHI 279, which is very sensitive to the antiproliferative effects of gamma-interferon [142]. The murine T cell line CTTL-20, which constitutively expresses IL-2 receptors [143], is used both as a model system and a bioassay to measure this important lymphokine. Tumor cell lines can also serve as a source for cytokines or other biological substances produced in limited quantities in normal populations of immune cells. For example, much of the early work deciphering the structure of immunoglobulins utilized immunoglobulin-secreting tumors as the source for these proteins. Tumor cell lines can also be used to study receptors and second-messenger systems.

Advantages of tumor cell lines. Tumor cell lines have many advantages over
normal cells for use in experimental studies. First, tumor cell lines are homogenous. This homogeneity has many advantages, especially when the presence of many cell types may give misleading results. Second, large numbers of cells can be obtained using cell lines. Third, stable cell lines can give very reproducible results. Finally, with the increased availability of molecular biological techniques, cell lines can now be 'made to order'.

There are many examples of discoveries that were first made in lymphoid cell lines that were later confirmed in purified populations of their normal counterparts. One example is the finding several years ago that several B cell lines contained NADPH-oxidase and were capable of generating a superoxide burst [144]. Recent studies were able to demonstrate that certain populations of normal B cells also contained a superoxide generating system [145].

Another example was the discovery by Hadjiagapiou and Sprecher that lymphocytes metabolized hydroxyeicosatetraenoic (HETE) acids by both beta- and alpha-oxidation [146]. The initial experiments used MOLT-4 T lymphoblasts to characterize the numerous new compounds made from $[^{3}H]15$-HETE and $[^{3}H]12$-HETE through these novel metabolic pathways. Later studies demonstrated that purified normal T lymphocytes metabolized $[^{3}H]15$-HETE in a similar manner.

Problems with tumor cell lines. Although tumor cells have many advantages as experimental models, there are drawbacks to their use. Care must be exercised in the application of results from tumor cell lines to their normal counterparts.
The literature abounds with examples of apparent discrepancies, including the finding that anti-Ig treatment results in the activation and subsequent proliferation of resting normal B cells, yet inhibits the spontaneous proliferation of several B cell lines [147]. For these reasons, it is desirable to also use normal cells to assess the usefulness of a tumor cell line model system.

In summary, tumor cell lines have been useful, and continue to play an important role in experimental immunology. Tumor cell lines have provided homogeneous populations of transformed cells in essentially unlimited quantities to allow the study of many immune cell populations. Because of purification problems, and/or the low numbers of these normal cell types found naturally, many of these normal cell types could not be studied without the use of tumor cell lines. Although many model systems using tumor cell lines are felt to be accurate representation of their normal counterparts, care must be exercised in the applications of results from studies using cancer cells.

B. PAF METABOLISM IN HUMAN LYMPHOCYTES

*Human lymphocytes metabolize PAF by deacetylation-transacylation.* Our results indicate that the lymphoid cell lines MOLT-4 and Raji rapidly metabolize PAF. Kinetic studies provided evidence that PAF is first deacetylated to lysoPAF, then acylated to form acylPAF (1-alkyl-2-acyl-GPC). Radioactivity was also found migrating with neutral lipids, which is consistent with phospholipase C (PLC)-
mediated metabolism of PAF. Since steps were not taken to characterize the neutral lipids produced, it is not known whether PAF, lysoPAF, or acylPAF (or all of the above) served as the substrate for PLC. However, the finding that the incubation of [³H]CPAF with Raji lymphoblasts did not result in a significant amount of [³H]neutral lipid produced, (by analogy) suggests that lysoPAF and/or acylPAF are probably serving as the major precursors of the [³H]neutral lipid seen with lymphocyte [³H]PAF metabolism.

The metabolism of PAF in purified populations of peripheral blood T lymphocytes was qualitatively similar, although less rapid, than that found in the lymphoid cell lines MOLT-4 and Raji. A possible explanation for this finding is that the spontaneously proliferating tumor cells are more metabolically active than their resting normal counterparts. Lymphocyte PAF metabolism was found to be temperature-dependent, with negligible metabolism at 4 °C.

Several lines of evidence indicate that the metabolism of PAF in purified T lymphocytes was not due to contamination by other cells. First, initial PAF metabolism studies used the T cell line MOLT-4. These homogeneous cultures of T lymphoblasts readily metabolized PAF. Second, the purified peripheral blood T lymphocytes were subjected to a number of tests to eliminate the possibility that they were heavily contaminated with other cell types. Since it is known that monocytes [148], neutrophils [16,60] and platelets [118,119] metabolize PAF, the purified T cells were assessed for the presence of these cells. The populations of
T lymphocytes purified using the T lympho-kwik procedure did not respond to mitogenic doses of the lectin PHA-p, suggesting that a significant accessory cell (monocytic) contamination was not present. This is in agreement with the findings of Clouse et al. [104]. Following the purification procedure, visualization with Wright-Giemsa stain indicated that a multinucleated cell (neutrophil) contamination was not present. Platelets were removed by 3-4 PBMC washings and by T Lympho-kwik. This results in a platelet contamination of less than 1 platelet/100 T-lymphocytes. According to Pieroni and Hanahan [118], platelets convert $5 \times 10^8$ M PAF to 1-alkyl-2-acyl-GPC at the rate of 42 pmol/min/10$^{10}$ cells. As shown in Figure 8, purified T-lymphocytes ($2.5 \times 10^6$) will convert 10 pmoles (20 % of 50 pmoles added) of PAF to 1-alkyl-2-acyl-GPC in 64 min. Assuming a contamination of 1 platelet/100 T-lymphocytes, approximately $2.5 \times 10^4$ platelets would be present in the incubation mixture. The amount of 1-alkyl-2-acyl-GPC formed by this number of platelets in 64 min would be less than 10 fmole, or 0.1 % of the total product. For these reasons, the metabolism of PAF by purified lymphocytes is probably not due to contamination.

Previous metabolic studies of PAF in other cell types indicate differences in the activities of the acetyl hydrolase and acyltransferase enzymes. For example, accumulation of the deacetylated intermediate lyso-PAF has been demonstrated in PAF catabolism studies in endothelial cells [149,150], fibroblasts [150] and alveolar macrophages [148], suggesting that these cells have a higher ratio of
acetyl hydrolase activity to acyltransferase activity. In contrast, the major metabolite of PAF metabolism in T-lymphocytes is 1-alkyl-2-acyl-GPC, with only a small amount of lyso-PAF present. This finding, which is similar to that found in platelets [118,119] and neutrophils [16,60] indicates that the acyltransferase activity is higher in T-lymphocytes.

**PMSF and PAF analogs inhibit MOLT-4 PAF metabolism.** The serine hydrolase inhibitor PMSF has been shown to inhibit the conversion of PAF to 1-alkyl-2-acyl-GPC in platelets [115,116]. This inhibition of PAF metabolism was thought to be due to inhibition of the acetyl hydrolase enzyme, as PMSF was shown to affect neither the acylation of lyso-PAF nor the uptake of PAF by platelets [116]. More recent work by Qian et al. [117] demonstrated that PMSF (1 mM) inhibited the activity of neonatal rat myocyte alkylacetetyl-GPC acetyl hydrolase and had no effect on alkyllyso-GPC transacylase activity. These studies also showed that treatment of intact cells with PMSF inhibited the metabolism of both PAF and lyso-PAF, suggesting that PMSF probably exerts its inhibitory effect on PAF metabolism by more than one mechanism. Our results indicate that PMSF pretreatment results in an inhibition of PAF metabolism in intact MOLT-4 lymphoblasts. Although treatment with PMSF did not result in an accumulation of lyso-PAF, which is consistent with its postulated inhibition of the acetyl hydrolase enzyme, caution must be exercised in interpreting PMSF effects on PAF metabolism in intact cells.
Treatment of MOLT-4 cells with the PAF receptor antagonists CV-3988 and Ro19-3704 resulted in a dose-dependent inhibition of the PAF metabolism. The dual findings that the PAF receptor antagonist BN52021 did not affect PAF metabolism, and that MOLT-4 cells did not test positive for the presence of PAF receptors, indicates that this effect is not mediated through interaction with a membrane PAF receptor. Because CV-3988 and Ro19-3704 are structural analogs of PAF, one possible mechanism by which these compounds could act would be by inhibiting PAF metabolizing enzymes.

In summary, the results of these studies show for the first time that PAF (1-alkyl-2-acetyl-GPC) is metabolized by T-lymphocytes in vitro to acylPAF (1-alkyl-2-acyl-GPC). This finding suggests a possible role for these cells in the inactivation of this biologically active phospholipid in vivo. This metabolism is temperature-dependent, and inhibited by PMSF and the PAF analogs CV-3988 and Ro19-3704.

C. IDENTIFICATION AND CHARACTERIZATION OF THE RAJI LYMPHOBLAST PAF RECEPTOR

In order to avoid binding artifacts due to ligand metabolism, we conducted the PAF binding assays at 4 °C, a temperature at which Raji lymphoblasts were shown to metabolize PAF at a very low rate. Raji lymphoblasts are not unique, as neutrophils have also been shown not to metabolize PAF at 4 °C [28].[^H]PAF
specific binding increased over time and reached equilibrium by 80 minutes. Control experiments under identical conditions utilizing 5 x 10^6 human neutrophils/ml also indicated that binding reached saturation by 80 minutes. PAF binding to Raji lymphoblasts was only partially reversible. Similarly, PAF binding to neutrophils [26-28] platelets [25,26,57-59] and monocytes [29] has been shown to be either irreversible or only partially reversible. Construction of saturation binding isotherms demonstrated that PAF specific binding increased with increasing concentrations of [\textsuperscript{3}H]PAF and the binding was saturable. Scatchard analysis revealed that Raji lymphoblasts expressed a single class of binding sites that have a dissociation constant (K_D) of 2.3 ± 0.3 nM. This dissociation constant found for Raji lymphoblasts is similar to reported values for human platelets [25,26,57-59] and is equal to [26,27] or somewhat higher than [28] reported values for human neutrophils. The number of binding sites was determined to be 17,800 ± 3,600/cell. Scatchard analysis of competition binding studies also revealed a single saturable high affinity site. A second type of binding site of very low affinity with essentially unlimited binding capacity was also found. This second type of PAF binding site has been demonstrated in other tissues [25,27] and is thought to be due to non-receptor cellular uptake. Although the data seem compatible with a model utilizing one high affinity binding site, they do not rule out the possibility of several classes of high affinity binding sites.

Evaluation of binding data by Scatchard analysis requires equilibrium binding
conditions. As PAF binding to Raji lymphoblasts was only partially reversible, true equilibrium conditions were not in operation. This problem of the use of Scatchard analysis under irreversible [³H]PAF binding conditions has been addressed recently by Kloprogge and Akkerman [57] and Valone [29] by determination of the kinetics of initial specific binding. Since these investigators demonstrated that the dissociation constants calculated in this manner were very close to those derived by assuming equilibrium binding conditions, our assumption of equilibrium binding provides a reasonable estimate.

PAF binding sites are functionally active. The [³H]PAF binding sites found on Raji lymphoblasts were shown to be functional PAF receptors, since PAF treatment resulted in intracellular calcium mobilization. The EC₅₀ calculated from the PAF dose-response curve (6.3 nM) was very close to the Kᵦ obtained from the binding studies (2.3 ± 0.3 nM), suggesting that the high-affinity PAF receptor mediated this response. The pattern of PAF-induced calcium changes in Raji lymphoblasts is similar to that observed in other PAF-responsive cells such as platelets [47-49] neutrophils [49] and monocytes [41,50]. The finding that removal of extracellular calcium ablated this response suggests that much the intracellular calcium mobilization is mediated through an external calcium channel. Raji cells are not unique in this regard, as PAF-induced intracellular calcium mobilization in platelets [47-49], neutrophils [49], monocytes [41,50] and PC-12 cells [51,52] is either partially or totally dependent upon the presence of extracellular calcium.
PAF antagonists inhibit PAF-induced calcium mobilization. The changes in [Ca^{2+}]_i induced by PAF treatment were blocked by CV-3988, CV-6209, alprazolam and BN52021, structurally different PAF receptor antagonists at doses which competed with[^3]H]PAF binding. The similarity of the concentrations of CV-6209 and alprazolam needed to inhibit 50% (IC_{50}) of[^3]H]PAF binding and PAF-induced calcium mobilization is further evidence that the increase in intracellular calcium concentration ([Ca^{2+}]_i) is receptor-mediated. In contrast, the biologically inactive [5] precursor/metabolite lysoPAF did not change baseline [Ca^{2+}]_i values, nor did it compete with[^3]H]PAF for binding to Raji lymphoblasts.

The Raji and platelet PAF receptors are similar. Several lines of evidence indicate that the PAF receptor expressed on Raji lymphoblasts is similar to that expressed on platelets. First, the PAF receptors on these two cell types have similar dissociation constants. Second, treatment of both Raji lymphoblasts and platelets with PAF results in intracellular calcium mobilization. Third, evidence from competition binding studies indicates the receptor antagonists Ro19-3704, CV-6209, CV-3988, BN52021 and alprazolam compete with PAF for binding to these two cell types with a similar pattern of potencies. Data from a recent study by Tahraoui et al. [151] comparing the IC_{50}s of these PAF receptor antagonists to compete with[^3]H]PAF for binding to, and the ability to inhibit PAF-induced aggregation in canine platelets, are listed in Table 8. These values are compared with the IC_{50}s of PAF receptor antagonists effects on PAF binding and PAF-
induced intracellular calcium mobilization. As illustrated in Table 8, the rank order of potencies of these antagonists are somewhat similar.

If the Raji lymphoblast PAF receptor is similar to other PAF receptors, this cell line could be a useful model system for future studies of the PAF receptor. The use of Raji lymphoblasts would have many advantages over the use of other normal cells that express PAF receptors. Raji is a stable cell line that expresses a large number (~18,000/cell) of PAF receptors. Since Raji is a tumor cell line, large numbers of these cells can be grown. The Raji lymphoblast PAF receptor is coupled to intracellular calcium mobilization, a phenomenon easily measured with calcium sensitive dyes such as fura-2. In summary, preliminary experiments indicate that the Raji PAF receptor is similar to other PAF receptors, notably the PAF receptor expressed on platelets. Raji lymphoblasts could be of use in further studies of the PAF receptor and would be an ideal source for the future isolation and molecular characterization of the receptor for this important autacoid.

The inhibition of PAF binding but not calcium response by calcium channel antagonists. Several studies have shown that the calcium channel antagonists verapamil and diltiazem inhibit [³H]PAF binding and PAF-induced aggregation in human platelets [53,54,152]. These results suggested that these compounds had PAF receptor antagonistic qualities. Our competition binding studies with verapamil, diltiazem and nimodipine indicated that these compounds inhibited [³H]PAF binding to the Raji lymphoblast PAF receptor. The finding that
The ability of known PAF receptor antagonists to compete with [3H]PAF for binding to or ability to inhibit PAF-induced increase in Raji [Ca\(^{2+}\)], or PAF-induced canine platelet aggregation are compared. Each value represents the mean ± S.E.M. of either the \(K_i\) for binding or \(IC_{50}\) for inhibition of either PAF-induced calcium mobilization or aggregation of Raji lymphoblasts or washed canine platelets [151], respectively.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Raji Ki (nM)</th>
<th>IC(_{50}) (nM)</th>
<th>Platelet Ki (nM)</th>
<th>IC(_{50}) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV-6209</td>
<td>2.3 (0.4)</td>
<td>3.5 (1.6)</td>
<td>10 (1.2)</td>
<td>8.6 (0.8)</td>
</tr>
<tr>
<td>Ro 19-3704</td>
<td>1.7 (0.3)</td>
<td>ND</td>
<td>36 (5)</td>
<td>357 (26)</td>
</tr>
<tr>
<td>CV-3988</td>
<td>142 (42)</td>
<td>ND</td>
<td>1397 (142)</td>
<td>3922 (112)</td>
</tr>
<tr>
<td>BN52021</td>
<td>376 (104)</td>
<td>ND</td>
<td>16,840 (1475)</td>
<td>1190 (63)</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>1840 (240)</td>
<td>7200 (3000)</td>
<td>3301 (555)</td>
<td>2872 (121)</td>
</tr>
</tbody>
</table>
nimodipine, a much more potent calcium channel blocker in many systems [56], was equipotent to the other compounds, suggests that these compounds were not exerting this effect on PAF binding by acting as voltage-sensitive calcium channel antagonists. In addition, unlike the PAF competitive receptor antagonists, diltiazem and verapamil did not inhibit PAF-induced calcium mobilization, even at doses that inhibited > 80% of [3H]PAF binding (results not shown). This finding suggests that these compounds do not inhibit Raji cell [3H]PAF binding by direct competition with the receptor. The type of inhibition (competitive, noncompetitive) can be assessed by conducting saturation binding isotherms in the presence of several concentrations of inhibitor, and subjecting the data to Scatchard analysis [109]. Unfortunately, these type of experiments, that could possibly confirm that these calcium channel antagonists are not true competitive PAF receptor antagonists, were not conducted. That diltiazem and verapamil competed with PAF for binding to both platelets and Raji lymphoblasts, and inhibited PAF-induced platelet aggregation, but not PAF-induced calcium response with Raji lymphoblasts would seem to be an apparent discrepancy. One possible explanation for this finding is that these compounds may not be specific competitive PAF receptor antagonists. Because platelet aggregation is a complex process, there are many sites at which compounds can inhibit PAF-induced platelet aggregation in addition to the ability to compete with PAF for binding to its receptor.
**CPAF is a metabolically stable lymphocyte PAF receptor agonist.** 1-hexadecyl-2-N-methylcarbamyl-GPC (CPAF) is a synthetic PAF analog that has been shown to be a metabolically stable PAF receptor agonist [121]. Because of the potential importance of such a compound in long-term studies of lymphocyte function, CPAF was tested in our system. Unlike PAF, \[^{3}H\]CPAF was shown not to be significantly metabolized by Raji lymphoblasts. CPAF competed with \[^{3}H\]PAF for binding to Raji lymphoblasts, and \[^{3}H\]CPAF was specifically bound to these cells. Scatchard plots of \[^{3}H\]CPAF saturation binding isotherms resulted in binding constants similar to those found for \[^{3}H\]PAF. These experimental findings indicate that CPAF acts at the PAF receptor. CPAF treatment resulted in an increase in Raji \([Ca^{2+}]\), similar to PAF, demonstrating that this stable PAF analog is a PAF receptor agonist. Since both PAF and CPAF treatment resulted in similar short-lived increases in \([Ca^{2+}]\), ligand metabolism probably is not responsible for the return of \([Ca^{2+}]\), to baseline levels within several minutes. The finding that CPAF and PAF had similar binding and calcium mobilization potencies is in contrast to the findings of O'Flaherty *et al.* [121], which indicated that CPAF was only 20% as potent as PAF in neutrophil binding and elastase release studies.

**The role of the PAF receptor in Raji lymphoblast function.** The physiological significance of the functional PAF receptor expressed on Raji lymphoblasts is not clear. The finding that Raji cells expressed high numbers of these PAF receptors prompted the search for an effect of pharmacological activation/inhibition of this
receptor on Raji lymphoblast function. The functional parameter examined in these cells was spontaneous proliferation, measured indirectly by $[^3H]thymidine incorporation studies. The resulting preliminary studies provided no evidence that the PAF receptor played a role in the proliferative process of this cell line.

D. EVIDENCE FOR A FUNCTIONAL PAF RECEPTOR IN OTHER B LYMPHOID CELL LINES

The demonstration of PAF receptors on Raji, an Epstein-Barr Virus (EBV)-immortalized B cell line from a Burkitt lymphoma [120] prompted the examination of other lymphoid cell lines for PAF receptors. Twelve other cell lines, both B and T cell lines, with and without the EBV genome, were assessed for the ability to specifically bind $[^3H]PAF$ and to respond to PAF treatment with an increase in $[Ca^{2+}]$. Five lymphoid cell lines in addition to Raji (P3HR-1, PB-1, Dakiki, BJAB, BJA/HR-1) specifically bound $[^3H]PAF$. Fura-2 fluorescence studies showed that three of these cell lines (P3HR-1, PB-1, Dakiki) responded to PAF treatment with an increase in $[Ca^{2+}]$. PAF treatment did not result in an increase of $[Ca^{2+}]$, above control values in cells that did not contain specific binding.

Both the B cell lymphoma line, BJAB, and the EBV-converted BJAB line, BJA/HR-1, contained specific PAF binding sites. However, treatment with PAF did not result in a change in $[Ca^{2+}]$, in either of these cell lines. We examined whether treatment of these cells with the ionophore Br-A23187 and aluminum
fluoride, a nonspecific activator of G-proteins [153] would result in increases in 
$[Ca^{2+}]_i$. Both of these stimuli resulted in an increase in the $[Ca^{2+}]_i$ of BJAB and 
BJA/HR-1, suggesting that both cell lines can be stimulated to produce a $[Ca^{2+}]_i$ 
increase (data not shown). Possible explanations for our finding that these two 
related cell lines specifically bind PAF but do not show an increase in $[Ca^{2+}]_i$ in 
response to PAF treatment are that the PAF-induced change in baseline $[Ca^{2+}]_i$ is 
too small to measure under our experimental conditions or that the PAF binding 
site in these cells is not coupled to intracellular calcium mobilization.

Although the number of lymphoid cell lines tested is small, several conclusions 
can be drawn from these studies. First, the presence of PAF binding sites is 
probably not related to the presence of the EBV genome. This is suggested by the 
fact that the B cell lymphoma BJAB does not contain EBV DNA [137] yet 
specifically binds PAF. The finding that cell lines (Daudi, B95-8, sfBT, CB-1) 
contain the EBV genome, yet do not express PAF receptors, also indicates a lack 
of connection between the presence of EBV genome and PAF receptor.

Second, the ability to synthesize PAF is not related to the presence of PAF 
receptors. The cell lines MOLT-4, CCRF-CEM, Daudi and Raji have been shown 
to synthesize PAF upon stimulation with the ionophore A23187 and the mitogenic 
lectin phytohemagglutinin [95]. Although all four of the cell lines synthesize PAF, 
only Raji contains PAF receptors. This finding suggests that the presence of the 
PAF receptor is not necessary for PAF synthesis to occur.
Third, the PAF receptor is not necessary for PAF metabolism to occur. Both MOLT-4 and Raji cell lines readily metabolize PAF by the deacetylation-reacylation pathway characteristic of neutrophils and platelets. Since our results indicate that MOLT-4 lymphoblasts do not express a PAF receptor, PAF can be metabolized independent of its receptor.

The finding that several lymphoid cell lines of B-cell origin have PAF receptors suggests that their normal counterparts may also express PAF receptors. To assess whether normal resting lymphocytes express functional PAF receptors, B and T lymphocytes were purified from PBMC by the use of Lympho-kwik, and tested for PAF-induced intracellular calcium mobilization. Preliminary experiments indicated that PAF treatment resulted in an increase in $[Ca^{2+}]_i$ in PBMC, but not purified resting populations of B or T lymphocytes (results not shown).

In summary, the results of these studies extended the earlier observation that Raji lymphoblasts express functional PAF receptors by describing other lymphoid cell lines that also contain PAF receptors. Preliminary studies indicate that resting B and T lymphocytes do not express a functional PAF receptor.

E. THE LYMPHOCYTE PAF RECEPTOR

Although several functional studies suggest T lymphocytes contain PAF receptors [98-100,102], we were unable to detect a PAF receptor on the two T cell
lines (MOLT-4, CCRF-CEM) tested. Experiments examining the role of PAF in
mitogen-stimulated peripheral blood mononuclear cell T lymphocyte proliferation
(see Appendix) demonstrate that both PAF agonists and PAF antagonists inhibit T
cell function. These results are consistent with previously reported studies
examining the effect of PAF on T cell function using the same model system
[98,99]. Although our results are similar, our interpretation that T cells probably
do not express a functional PAF receptor contrasts greatly with conclusions drawn
from previously reported studies. Several lines of evidence lead to our
interpretation. First, the concentrations of PAF or PAF receptors antagonists
necessary to affect T cell function are very high. In other systems, PAF exerts its
effects at picomolar and nanomolar levels, concentrations consistent with the
affinity of the PAF receptor. However, micromolar levels of PAF and PAF
receptor antagonists are necessary to inhibit T cell function. Metabolism probably
cannot account for the need for these high concentrations, especially since the
metabolically stable PAF agonist CPAF only affects T cell function at levels
approximately three orders of magnitude higher than its affinity for the PAF
receptor.

The inability of specific PAF receptor antagonists to reverse the effects of PAF
and CPAF on T cell function also suggests that this response is not mediated by
the PAF receptor. In particular, our finding that BN52021 inhibits PAF-induced
$[\text{Ca}^{2+}]$, mobilization and competes with PAF for binding to Raji lymphoblasts, yet
does not by itself affect, or reverse the effects of PAF agonists or antagonists on T cell function, is further evidence that the PAF receptor is not involved in this phenomenon.

If these PAF agonists and PAF antagonists do not exert their effects on T cell function via interaction with a PAF receptor, then one would expect that these compounds would be able to affect PAF receptor-negative cells. Our finding that PAF metabolism in the PAF receptor-negative MOLT-4 cell line is inhibited by the PAF receptor antagonists CV-3988 and Ro19-3704 (Figure 9), and the PAF receptor agonist CPAF (*results not shown*) demonstrates that these compounds may exert effects independent of the PAF receptor. Interestingly, the potencies of Ro19-3704, CV-3988 and BN52021 are similar with respect to both the inhibition of MOLT-4 PAF metabolism (Figure 9) and inhibition of mitogen-stimulated T cell proliferation (Figure 42). These similarities indicate the possibility that the effects of PAF-like compounds on T cell function may be due to effects on phospholipid metabolism or non-specific membrane effects.

F. CONCLUSIONS

The data presented in this dissertation indicate that human lymphocytes metabolize PAF, and certain B lymphoid cell lines express functional PAF receptors. Lymphocytes readily metabolize PAF by the deacetylation-reacylation pathway characteristic of neutrophils and platelets. The B lymphoid cell line Raji
expressed a large number of high affinity PAF binding sites. These binding sites were shown to be functional receptors as PAF treatment resulted in an intracellular calcium mobilization. PAF binding and PAF-induced calcium mobilization were inhibited by known PAF receptor antagonists. Raji was not unique, as other B lymphoid cell lines also expressed functional PAF receptors.

G. SIGNIFICANCE AND FUTURE EXPERIMENTS

The discovery that certain B lymphoid cell lines express functional PAF receptors has important implications. Two potentially important areas for future studies are the role of the PAF receptor in B cell function, and the possible clinical implications of PAF receptors expressed on leukemias.

The role of the PAF receptor in B cell function.

The finding that several B cell lines expressed PAF receptors suggests that normal B cells may also express PAF receptors. Demonstration that normal B cells express a PAF receptor at some stage of development/activation is an important first step in the delineation of the role of this phospholipid in B cell function.

The existence of PAF receptors on B cells can be examined by looking for $[^3H]PAF$ specific binding and PAF effects on $[Ca^{2+}]$ in purified B cell populations. Because larger quantities of cells are needed to conduct binding studies than fura-2 fluorescence studies, preliminary experiments can utilize the latter. Both resting
and activated populations of B lymphocytes can be tested for PAF-induced intracellular calcium mobilization. The B cell populations utilized in these experiments can be purified either from blood, or other sources such as spleen or tonsils. To confirm that any increase in $[\text{Ca}^{2+}]_i$ seen following PAF treatment is actually due to the presence of a PAF receptor, lysoPAF and PAF receptor antagonists can be used. Once conditions for the expression of a lymphocyte PAF receptor (based on fura-2 studies) are established, binding studies can be used to further characterize the lymphocyte PAF receptor. Both fura-2 and binding studies can be used not only to characterize a possible PAF receptor on normal lymphocytes, they can be used to compare this PAF receptor with that found on Raji lymphoblasts. This information could be of use in the assessment of the Raji PAF receptor as a model to study the lymphocyte PAF receptor.

The possible role of PAF on B cell function can be explored by testing the effect(s) of CPAF and PAF receptor antagonists on B cell functions utilizing several model systems. Model systems which can be used to examine B cell activation and subsequent proliferation include B cell stimulation with the mitogens Streptococcus A Cowan (SAC) [154,155], anti-immunoglobulin [156,157] or phorbol esters [158,159]. Experiments could be designed to measure immunoglobulin production in addition to blast-transformation.

Finding a functional PAF receptor expressed on B lymphocytes and the delineation of the role of this phospholipid in B cell functions could have important
clinical implications. There are many pathological conditions due to either a hypoactive or hyperactive humoral immune response. Many of these conditions are treated pharmacologically by nonspecific immunomodulators such as corticosteroids. An understanding of the natural regulatory mechanisms of the humoral immune response can be used in the development of more selective therapeutic strategies.

Clinical implications of PAF receptors expressed on leukemias

Our discovery that several leukemic cell lines express functional PAF receptors raises an interesting possibility that the PAF receptor found on these cells may have clinical implications. Inasmuch as recent evidence suggests that PAF is involved in the normal process of tumor cytotoxicity, one might expect that PAF receptor-positive leukemias could be managed pharmacologically with PAF-like compounds, or by methods that enhance the patients own immune system.

The role of PAF in tumor surveillance. Large granular lymphocytes (LGLs) are a class of lymphocytes that are thought to be involved in tumor surveillance [160]. Several lines of evidence suggest that PAF is involved in LGL function. LGLs synthesize and release PAF in response to such stimuli as anti-immunoglobulin and the ionophore A23187 [96]. Recently, the PAF receptor antagonist BN52021 was shown to inhibit tumoricidal activity of LGLs against a myeloid target line [161]. BN52021 was most potent when it was preincubated with the target cells rather than the effector cells, suggesting that BN52021 exerted this antitumoricidal effect
by interaction with PAF receptors on this cell line. Although the K652 myeloid
target cell line was not tested for PAF receptors, many other myeloid cell lines
express PAF receptors. Further evidence that BN52021 was exerting this effect
by acting as a PAF receptor antagonist was the demonstration that other PAF
receptor antagonists exerted the same anti-tumoricidal effect. BN52020, a
compound structurally similar to BN52021, but not having anti-PAF qualities [66],
did not have any effect on LGL-mediated cytotoxicity. These findings suggest that
PAF may be involved in the cytotoxic process of LGLs.

Future experiments can assess the clinical implications of PAF receptors
expressed on leukemias, and whether PAF is involved in LGL-mediated tumor
cytotoxicity. LGL cytotoxicity assays can be conducted using as target cells either
PAF receptor positive (Raji, P3HR-1) or negative (Daudi) cell lines. The ability
of PAF receptor agonists (CPAF) and PAF receptor antagonists (CV-6209,
BN52021, alprazolam) to modulate LGL cytotoxicity can also be tested. To show
that the effect(s) are due to effector or target cells, one could preincubate drugs
with either of these two populations, wash, then put them together and measure the
resultant cytotoxicity response. These types of experiments can also be conducted
using either monocytes or macrophage cell lines as effector cells.

The leukemia PAF receptor as a tumor marker. Patient leukemias can be
tested in a similar manner as the cell lines in Table 6. The presence/absence of
the PAF receptor could also be compared with other cell markers (surface
immunoglobulin, etc.) or clinical parameters (stage, outcome, etc.).

The present state of cancer chemotherapy is quite primitive, as most of the agents used to manage cancers are not very selective. An understanding of the natural process of tumor surveillance could probably result in more rational therapy. Both PAF receptor agonists/antagonists and PAF receptor positive/negative cell lines would be useful tools in the delineation of the role of PAF and the PAF receptor in tumor immunity.

Like many discoveries, the significance of the discovery that certain B lymphoid cell lines express PAF receptors is dependent upon the results of future studies. These cell lines could serve many roles, from model systems to study PAF receptor expression, to a source of PAF receptors to allow receptor isolation and cloning. Although preliminary studies indicated that resting B cells do not express PAF receptors, the finding that four B cell lines contained functional PAF receptors raises the very distinct possibility that B cells express a PAF receptor at some stage of activation/differentiation.
A. SYNTHESIS OF CPAF

In view of the finding that human lymphocytes readily metabolized PAF, a more metabolically stable PAF receptor agonist was felt to be a useful tool in the elucidation of the effect of PAF on lymphocyte function. 1-O-alkyl-2-N-methylcarbamyl-GPC (CPAF) is a recently discovered PAF analog that contains a carbamoyl moiety instead of an acetate at the sn-2 position [121]. This modification makes CPAF unsusceptible to hydrolysis by acetylhydrolases or PLA₂. CPAF was shown to bind to, and behave as a PAF agonist in studies with human neutrophils [121]. Because CPAF was not commercially available, the synthesis of CPAF was undertaken based on the methods established in the laboratory of Piantadosi [121,162]. Briefly, CPAF was synthesized by treatment of lysoPAF (1-O-hexadecyl-2-(R)-lyso-GPC, Sigma Chemical Co. St. Louis MO.) with methyl isocyanate (Aldrich Chemical Co., Milwaukee, WI.) under anhydrous conditions.

*Preparation of CPAF.* 1-O-hexadecyl-2-lyso-GPC (10 mg) was first converted to its hydrochloride salt by treatment with methanol/chloroform/concentrated HCl
(2:1:0.1 v/v). Extracted material was then blown to dryness over a stream of nitrogen, and allowed to dry overnight under a vacuum pump. The residue was then dissolved in 1 ml dimethylformamide (Aldrich) and treated with 0.2 ml methyl isocyanate (Aldrich). After a 5 hr incubation at 50 °C, the reaction mixture was evaporated by nitrogen. The reaction mixture was then purified by TLC on LK6D plates (Whatman Inc., Clifton, NJ) using a basic solvent system containing chloroform/methanol/ammonium hydroxide (70:35:7 v/v). The reaction mixture was visualized under UV light by spraying the plates with 0.1% (w/v) 2',7'-dichlorofluorescein in ethanol. The zone corresponding to a standard of CPAF (Rf = 0.30) generously provided by Dr. Wykle of Bowman Gray, was scraped and the CPAF extracted from silica gel by the method of Bligh and Dyer [108].

Phosphorus analysis to quantitate CPAF. The concentration of CPAF was determined by phosphorus analysis utilizing a modification of the method of Rouser et al. [163]. Briefly, samples to be assayed were placed in 16 x 125 mm borosilicate test tubes, and the solvent was evaporated by a stream of nitrogen. The dried residue was reconstituted in 0.5 ml of distilled water. After vortexing, 0.65 ml of 72% (w/v) perchloric acid was added with gentle mixing. The samples were then heated at 180 °C for 1 hour to allow digestion. After allowing the tubes to cool, 3.3 ml of water was added. After vortexing, 0.5 ml of 2.5% (w/v) ammonium molybdate and 0.5 ml of 10% (w/v) ascorbic acid were added. The tubes were then heated at 100 °C for 10 minutes. The absorbance (at 810 nm) of
the resulting color change was determined using a Beckman DU-50 Spectrophotometer. KH$_2$PO$_4$ standards (20 - 200 nmoles) were used to construct a standard curve to allow conversion of absorption values to nanomoles of phosphorus.

Structure and purity determination. The purity of CPAF was assessed by TLC in chloroform/methanol/ammonium hydroxide (70:35:7 v/v). The finding of a single spot after exposing the plate to iodine vapor was taken as an indication of high purity. The structure was confirmed by gas chromatography-mass spectrometry of the corresponding tertiary-butyl dimethyl silane (TBDMS) derivative. Briefly, ~200 µg of either CPAF or PAF were placed in a siliconized tube and the solvents removed by a stream of nitrogen. One ml of diethyl ether was added to the dried residue and the tube was vortexed. Approximately 20 U of phospholipase C in 1 ml of buffer (consisting of 0.1 M tris(hydroxymethyl)aminomethane, 10 mM CaCl$_2$, pH = 7.4) was then added. A microstirring bar was then added, the tube was capped, and stirred for at least 4 hours. The resulting neutral lipid was extracted by addition of 5 ml of additional diethyl ether. The ether layer was transferred to another siliconized tube and the solvent was removed by a stream of nitrogen. The dried residue was reconstituted in 500 µl of chloroform and transferred to a siliconized Pierce vial. The chloroform was removed by a stream of nitrogen, and the residue reconstituted in 50 µl of acetonitrile. The neutral lipid was derivatized by reaction with 50 µl of
N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (Pierce Chemical Co., Rockford, IL.) for 1 hour at 50 °C. The solvents were removed by a stream of nitrogen, and the dried residue reconstituted in isooctane.

Gas chromatography was carried out on a Varian Vista 6000 gas chromatograph equipped with a 6 ft x 2 mm i.d. glass column packed with 1% SP-2100 on 100/200 mesh Supelcoport (Supelco, Bellefonte, PA.). Helium, 30 ml/min, was the carrier gas and the temperature of the injector and detector were maintained at 220 °C and 250 °C, respectively. The temperature of the oven was 210 °C. Equivalent chain length (ECL) values were determined from retention times of a series of saturated methyl esters (18:0 - 24:0). The ECL of the tBDMS derivatives of PAF and CPAF were > than 24.

Mass spectrometry was carried out with a Hewlett-Packard model 5970A mass selective detector and a 5790 gas chromatograph, under the supervision of Dr. Howard Sprecher (O.S.U.). The oven contained a 30 m x 0.25 mm i.d. DB-1 capillary column (Alltech Associates, Deerfield, IL). All injections were done in the splitless mode with an oven temperature of 70 °C. The oven was programmed at 30 °C/min to a final temperature of 260 °C. Both total ion current and m/z ions corresponding to M - 57 ions (415 and 430, for PAF and CPAF tBDMS derivatives, respectively) were monitored.

The mass spectrum of the tBDMS-ether of 1-hexadecyl-2-acetyl-glycerol (Figure 37) had ions at m/z = 415 (34%), 355 (44%) and 117 (100%). This
spectrum is similar to that found by Satouchi et al. [164]. The ion m/z = 415 is the result of a loss of tertiary butyl group (M - 57); m/z = 355 is the rearrangement ion formed from the hexadecyl sn-1 group; m/z = 117 is the rearrangement ion formed from the acetate sn-2 group.

Although the mass spectra of the tBDMS-ether of 1-hexadecyl-2-N-methylcarbamyl-glycerol has not been published, the cleavage pattern expected would be similar to the analogous PAF derivative. The expected ions would include: m/z = 430 (M - 57); m/z = 132 (rearrangement ion formed from N-methyl carbamyl sn-2 group); and m/z = 355 (rearrangement ion formed from the hexadecyl sn-1 group).

The mass spectrum of the tBDMS-ether of our compound (Figure 38) had ions at m/z = 430 (41%), 355 (8%) and 132 (100%). Based on the methodology, TLC and mass spectrometry, the compound synthesized was 1-hexadecyl-2-N-methylcarbamyl-GPC (CPAF).
FIGURE 37. THE MASS SPECTRUM OF TBDMS-ETHER OF 1-HEXADECYL-2-ACETYL-GLYCEROL.

The tert-butyltrimethylsilyl-ether of 1-hexadecyl-2-acetyl-glycerol was prepared from PAF (1-hexadecyl-2-acetyl-GPC) and the mass spectra obtained as described in Appendix A.
FIGURE 38. THE MASS SPECTRUM OF TBDMS-ETHER OF 1-HEXADECYL-2-N-METHYLCARBAMYL-GLYCEROL.

The mass spectrum of the tert-butylidimethylsilyl-ether of the neutral lipid of the CPAF synthesized as described in Appendix A was obtained as in Figure 37.
B. THE EFFECT OF PAF AND PAF RECEPTOR ANTAGONISTS ON MITOGEN-STIMULATED T LYMPHOCYTE PROLIFERATION

As indicated in the Introduction, the effect of PAF on mitogen-stimulated T lymphocyte activation and subsequent proliferation is controversial. In three-day cultures of PBMC stimulated with a mitogenic lectin, PAF has been shown to increase [100], decrease [98,99] or have no effect [101] on T cell proliferation as assessed by $[^{3}H]$TdR incorporation. In view of our finding that T lymphocytes readily metabolize PAF, more stable compounds with PAF-like or anti-PAF activities would be more appropriate tools for the determination of PAF effects in this model system. Thus, utilizing the methodology established in our laboratory by Dr. George Cox [113], PAF, CPAF, and PAF receptor antagonists were tested for the ability to modulate PBMC proliferation induced by the mitogenic lectin PHA-p.

1. THE EFFECT OF THE MITOGENIC LECTIN PHA-p ON LYMPHOCYTE PROLIFERATION

Initial experiments were designed to determine the optimal and suboptimal concentrations of PHA-p for lymphocyte proliferation. As shown in Figure 39, treatment of PBMC cultures with PHA-p resulted in an increase in lymphocyte proliferation. The concentration of PHA-p necessary to elicit an optimal response was approximately 1 $\mu$g/ml. Because the objective of these experiments was to
**FIGURE 39. THE EFFECT OF PHA-p ON LYMPHOCYTE PROLIFERATION.**

Various concentrations of the mitogenic lectin phytohemagglutinin (PHA-p) were added to cultures containing 1 x 10^5 PBMC. The resultant lymphocyte proliferation was detected after a 72 hour incubation and a 16 hour terminal pulse with [3H]Tdr, as described in *Methods*. Each value represents the mean ± S.E.M. of triplicate cultures from one representative experiment.
detect either a possible increase or decrease in lymphocyte proliferation due to drug treatment, a dose of PHA-p which resulted consistently in a suboptimal response was utilized. For the majority of experiments this dose of PHA-p was 0.25 \( \mu \text{g/ml} \), although a few experiments utilized 0.5 \( \mu \text{g/ml} \) as the suboptimal dose used.

B. THE EFFECT OF PAF, CPAF AND PAF ANTAGONISTS ON MITOGEN-STIMULATED LYMPHOCYTE PROLIFERATION

To determine the effect of PAF on lymphocyte proliferation, various concentrations of PAF (either dissolved in ethanol, DMSO or 2.5 mg/ml BSA in RPMI 1640) were incubated with PBMC along with mitogen. As demonstrated in Figure 40, PAF treatment did not affect lymphocyte proliferation at non-toxic doses (less than 1 \( \mu \text{M} \)).

The effects of the more metabolically stable PAF agonist CPAF, and PAF receptor antagonists CV-3988, Ro19-3704 and BN52021 on lymphocyte proliferation were next assessed by addition of these compounds to mitogen-stimulated PBMC cultures. CPAF did not affect lymphocyte proliferation until micromolar concentrations, where it exerted a suppressive effect (Figure 41). Ro19-3704 and CV-3988 treatment also resulted in a decrease in lymphocyte proliferation (Figure 42). However, BN52021 (up to 100 \( \mu \text{M} \)) did not affect mitogen-stimulated lymphocyte proliferation.
FIGURE 40. THE EFFECT OF PAF ON T LYMPHOCYTE PROLIFERATION.

Various concentrations of PAF were added to PBMC cultures stimulated with 0.25 μg/ml of PHA-p and the resultant proliferation was assessed as described in Figure 39. Each point represents the mean ± S.E.M. of triplicate cultures of a representative experiment of 5 conducted. The PAF was introduced with either 2.5 mg/ml BSA or DMSO (< 0.5%), which did not affect lymphocyte proliferation. Treatment of unstimulated PBMC cultures with PAF concentrations less than 1 μM did not affect cellular viability as assessed by trypan blue dye. However, treatment with 1 μM PAF resulted in a marked decrease in cellular viability.
FIGURE 41. THE EFFECT OF CPAF ON T LYMPHOCYTE PROLIFERATION.

Various concentrations of the PAF receptor agonist CPAF were added to PBMC cultures stimulated with PHA-p as described in Figure 40. Each point represents the mean ± S.E.M. of triplicate cultures of a representative experiment of 3 conducted.
FIGURE 42. THE EFFECT OF PAF RECEPTOR ANTAGONISTS ON T LYMPHOCYTE PROLIFERATION.

Various concentrations of the PAF receptor antagonists Ro19-3704 (■), CV-3988 (●), or BN52021 (▲) were added to PBMC cultures stimulated with PHA-p as described in Figure 40. The inhibition of proliferation (as assessed by [³H]Tdr incorporation) induced by the drug was expressed relative to control with vehicle (0.5% ethanol or DMSO). Each point represents the mean ± S.E.M. with n = 5 - 9.
To evaluate whether the antimitogenic effects of CPAF and Ro19-3704 were due to interaction with a PAF receptor, BN52021 was incubated along with CPAF or Ro19-3704. As shown in Figure 43 and Figure 44, BN52021 did not affect the suppression of mitogen-stimulated PBMC proliferation induced by either the Raji lymphoblast PAF receptor agonist (CPAF) or antagonist (Ro19-3704). Low doses of CPAF also did not affect the Ro19-3704-induced decrease in lymphocyte proliferation (Figure 45). Inasmuch as all of the above compounds competed with \[^3H\]PAF for binding to the Raji lymphoblast PAF receptor, these findings suggest that the antimitogenic effects of Ro19-3704, CV-3988 and CPAF are probably not due to interaction with a PAF receptor. The observed suppression of lymphocyte proliferation was not due to a cytotoxic effect as the concentrations of the above compounds used did not result in an increase in cytotoxicity in unstimulated PBMC cultures, as measured by trypan blue dye exclusion.

**Characterization of the anti-proliferative effects of Ro19-3704.** Because Ro19-3704 was more potent than the other PAF analogs, experiments were designed to further characterize the immunosuppressant properties of this compound. To determine the time-dependence of the observed suppression, triplicate PBMC cultures were stimulated with PHA-p and several concentrations of Ro19-3704 were added to individual cultures at various times thereafter. All of the cultures were pulsed with \[^3H\]TdR at 72 hours and harvested after an additional 16 hours of incubation as outlined in *Methods*. Table 9 and Figure 46 demonstrate that
FIGURE 43. THE EFFECT OF EXOGENOUS BN52021 ON CPAF INHIBITION OF T LYMPHOCYTE PROLIFERATION.

PBMC stimulated with PHA-p were incubated with either vehicle (DMSO) or 100 μM BN52021 ± 2.5 μM CPAF (shaded bar graphs) as described in Figure 41. Each bar graph represents the mean ± S.E.M. of triplicate cultures from one representative experiment of two conducted.
FIGURE 44. THE EFFECT OF EXOGENOUS BN52021 ON RO19-3704 INHIBITION OF T LYMPHOCYTE PROLIFERATION.

PBMC stimulated with PHA-p were incubated with either vehicle (DMSO) or 100 μM BN52021 ± 0.5 μM Ro19-3704 (shaded bar graphs) as described in Figure 42. Each bar graph represents the mean ± S.E.M. of triplicate cultures from one representative experiment of three conducted.
FIGURE 45. THE EFFECT OF EXOGENOUS CPAF ON RO19-3704 INHIBITION OF T LYMPHOCYTE PROLIFERATION.

PBMC stimulated with PHA-p were incubated with either vehicle (DMSO) or 100 nM CPAF ± 0.25 μM Ro19-3704 (shaded bar graphs) as described in Figure 41 and Figure 42. Each bar graph represents the mean ± S.E.M. of triplicate cultures from one representative experiment of two conducted.
**TABLE 9. THE TIME- AND DOSE-DEPENDENCE OF THE INHIBITION OF PHA-P-INDUCED T CELL PROLIFERATION BY RO19-3704.**

Various concentrations of Ro19-3704 were added to triplicate cultures of PHA-p-stimulated PBMC at different times, and the proliferation detected as described in Figure 39. The values represent the mean ± S.E.M. of the percent inhibition of PHA-p-induced proliferation with n = 4. A graphical representation of the time-dependence of 0.25 μM Ro19-3704 is shown in Figure 46.

<table>
<thead>
<tr>
<th>Time of Addition (hours post-mitogen)</th>
<th>R0193704 Concentration ( \text{(\mu M)} )</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>0</td>
<td>16 ± 7</td>
</tr>
<tr>
<td>24</td>
<td>53 ± 10</td>
</tr>
<tr>
<td>48</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>72</td>
<td>24 ± 10</td>
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</tbody>
</table>
FIGURE 46. THE EFFECT OF TIME OF ADDITION ON 0.25 μM RO19-3704 INHIBITION OF PHA-P-INDUCED T CELL PROLIFERATION.

0.25 μM Ro19-3704 was added to triplicate cultures of PBMC at different times as described in Table 9. Each point represents the mean ± S.E.M. of the percent inhibition of PHA-p-induced proliferation with n = 4.
Ro19-3704 was most efficient at suppressing PHA-p-induced PBMC proliferation when added between 24 and 48 hours of culture. Addition of Ro19-3704 at all times tested resulted in a decrease in lymphocyte proliferation.

Exogenous interleukin-2 does not reverse Ro19-3704 effects on lymphocyte proliferation. The activation and subsequent proliferation of PBMC induced by lectin mitogens is dependent on the endogenous production and secretion of lymphokines such as interleukin-2 (IL-2) [165]. Accordingly, one possible mechanism of action of the observed Ro19-3704 effects could be due to inhibition of endogenous IL-2 production/secretion. The effect of exogenous IL-2 administration on Ro19-3704-induced suppression of mitogen-stimulated PBMC proliferation was tested by addition of either 2% or 4% IL-2 (16 and 32 U/ml, respectively) at the beginning of the PBMC cultures. As illustrated in Figure 47, although exogenous IL-2 treatment resulted in an increase in PHA-p-stimulated lymphocyte proliferation, it did not overcome Ro19-3704 effects. The results of these experiments suggest that Ro19-3704 does not act solely by inhibition of IL-2 synthesis/secretion.

3. CONCLUSION

In summary, these preliminary experiments indicate that Ro19-3704, CV-3988 and CPAF inhibit mitogen-stimulated PBMC. Further studies failed to provide evidence that these PAF analogs act by interaction with a PAF receptor similar to
FIGURE 47. THE EFFECT OF EXOGONOUS IL-2 ON RO19-3704 INHIBITION OF T LYMPHOCYTE PROLIFERATION.

Various concentrations of interleukin-2 (IL-2) were added to PHA-p-stimulated PBMC cultures in the presence of 0.5 μM Ro19-3704 (■) or ethanol control (▲). The resultant proliferation was detected as in Figure 39. Each value represents the mean ± S.E.M. of triplicate cultures from a representative experiment of 3 conducted.
9that found in Raji lymphoblasts. Unlike the known immunosuppressive agents
cyclosporine [166] and 8-methoxypsoralen [113,167], time-dependence studies
revealed that Ro19-3704 exerted its maximal effects when added at 24 - 48 hours
post-mitogen treatment. The finding that exogenous IL-2 did not overcome the
suppressive effects of Ro19-3704 suggests that this compound does not act solely
by the inhibition of the production or secretion of this lymphokine.

Recent studies indicate that alkyl-lyso-phospholipids (ALPs), a recently
discovered class of synthetic ether phosphatidylcholines, have antiproliferative
activity [168]. The mechanism of action of these compounds is a matter of
speculation. However, both ALPs and neutral lipids prepared from these
compounds inhibit the ability of diacylglycerol to activate PKC [169-171]. The
structures of several first generation ALPs are shown in Figure 48. Comparison
of Figures 1, 4 and 48 demonstrate that CV-3988, Ro19-3704, CPAF, and PAF
resemble ALPs structurally. This structural similarity suggests the possibility that
the PAF analogs shown to inhibit mitogen-stimulated T lymphocyte proliferation
may act as ALPs.
FIGURE 48. THE STRUCTURE OF SOME ALKYL-LYSOPHOSPHOLIPID DERIVATIVES (ALPs) OF THE FIRST GENERATION [168]
REFERENCES


