

CHARACTERIZATION OF PLATELET ACTIVATING FACTOR
USING FORSKOLIN AND METHYLCARBAMYL PAF

by

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ABSTRACT

Platelet activating factor is an important mediator of septic shock and inflammation. The action of PAF on target cells is mediated through specific membrane bound receptors. PAF binding to receptors initiates a cascade of biochemical signalling events leading to the aggregation of platelets or the activation of other target cells. In this work we characterized the PAF-receptor physiochemical properties in platelets and investigated its interaction with signalling systems. The effect of forskolin on PAF receptor binding was studied. Studies were also conducted towards elucidation of the nature of the PAF receptor. In this direction the binding of the stable PAF analog 1-O-hexadecyl-2-O-(methylcarbamyl)-sn-glycero-3-phosphocholine (MC-PAF), a compound with potential uses in PAF receptor purification and antibody production was evaluated.

Forskolin is commonly used in the investigation of the effects of stimulated adenylyl cyclase activity in cells. In the first project the effect of forskolin on platelet activating factor receptor was explored. Rabbit platelets treated with forskolin prior to PAF binding resulted in a 30-40% decrease in binding which translated to a change in receptor number on the cell surface. This decrease in PAF binding caused by forskolin was concomitant with a decrease in platelets' physiological response to PAF. However, the forskolin induced decrease in PAF binding was not a consequence of cAMP formation as the addition of a cAMP analog could not mimic the actions of forskolin. Additionally, the inactive analog of forskolin, dideoxyforskolin, which does not activate adenylyl cyclase, also reduced PAF binding to its receptor. Reduction of PAF binding by forskolin and dideoxyforskolin was also demonstrated in isolated platelet

membranes. The action of forskolin was also found to be independent of G-protein involvement. We therefore concluded the PAF receptor may be regulated by several factors that are triggered by forskolin. These elements are independent of adenylyl cyclase involvement.

In the second project MC-PAF was evaluated using rabbit platelets. MC-PAF was found to be approximately 3-5 fold less active in causing platelet aggregation and serotonin release. Aggregation caused by MC-PAF was completely inhibited by WEB 2086, a PAF receptor antagonist. Ligand binding studies revealed that MC-PAF binds with approximately two-fold lower affinity than PAF to the PAF receptor ($K_d=1.47$ and 0.62 nM for MC-PAF and PAF, respectively). Studies in anesthetised rats showed that both PAF and MC-PAF caused a similar reduction in blood pressure without changes in either heart rate or EKG parameters. The susceptibility of PAF and MC-PAF to hydrolysis by serum acetylhydrolases was investigated. PAF was found to be fully hydrolyzed after 5 min in rabbit serum while MC-PAF was not degraded significantly after a 1 hour exposure. These studies suggested that MC-PAF is a full agonist of the PAF receptor, both *in vitro* and *in vivo*, and is more stable than PAF against serum acetylhydrolases, thus making MC-PAF a useful compound for purifying the PAF receptor as well as raising PAF antibodies.

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ABBREVIATIONS

| | |
|-------------------------------|---|
| A3 | N-(2-aminoethyl)-5-chloronaphthalene-1-sulfonamide hydrochloride |
| ADP | adenosine diphosphate |
| AlF ₄ ⁻ | fluoroaluminate |
| BSA | bovine serum albumin |
| cAMP | cyclic adenosine monophosphate |
| CPT-cAMP | 8-(4-chlorophenylthio)-adenosine 3'-5'-cyclic monophosphate |
| DAG | diacylglycerol |
| G _i | inhibitory guanine nucleotide binding protein |
| G-protein | guanine nucleotide binding protein |
| G _p | phospholipase C linked guanine nucleotide binding protein |
| G _s | stimulatory guanine nucleotide binding protein |
| GTP | guanosine triphosphate |
| IP ₃ | inositol-1,4,5-triphosphate |
| IBMX | 3-isobutyl-1-methyl-xanthine |
| Lyso-PAF | 1-0-alkyl-sn-glycero-3-phosphocholine |
| MC-PAF | 1-O-hexadecyl-2-O-(methylcarbamy)-sn-glycero-3-phosphocholine |
| PAF | platelet activating factor/1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine |
| PIP ₂ | phosphatidyl inositol-4,5-bisphosphate |
| PKA | cAMP dependent protein kinase A |
| PKC | calcium dependent protein kinase C |
| PLA | phospholipase A ₂ |

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Chapter 1

Literature Review

1.1 Introduction

Platelet activating factor (PAF) was first described as a soluble factor that caused the aggregation of platelets in IgE-sensitized rabbit basophils in a calcium and temperature dependent process (1). Since its discovery it has been demonstrated that PAF is synthesized in a wide number of cell and tissues. Furthermore, studies have implicated PAF as an important mediator in a number of diseases including asthma and septic shock.

1.2 PAF Structure

The structure of PAF is defined as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (2,3) (figure 1.1). The naturally occurring enantiomer of PAF is primarily a mixture of C₁₆ and C₁₈ ether chains at the first carbon and is in the (R) configuration at the second carbon. Both these structural features of PAF are essential for maximal stimulatory activity. Reducing chain length at the first carbon or changing isomerism at the second carbon can result in up to a thousand fold drop in activity (4,5).

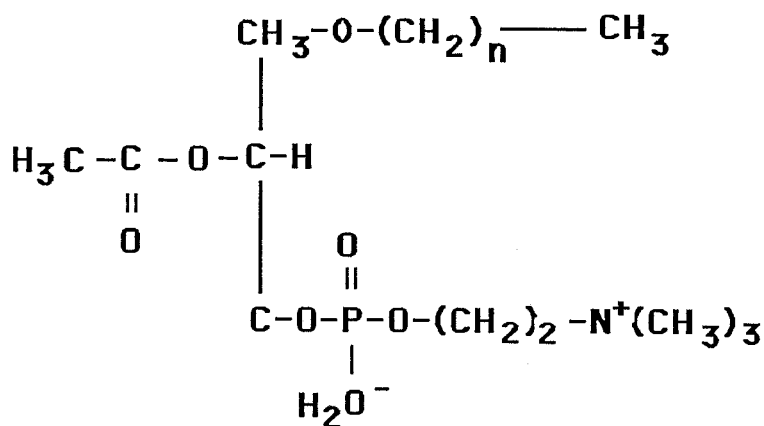


Figure 1.1 PAF structure, n=14-17.

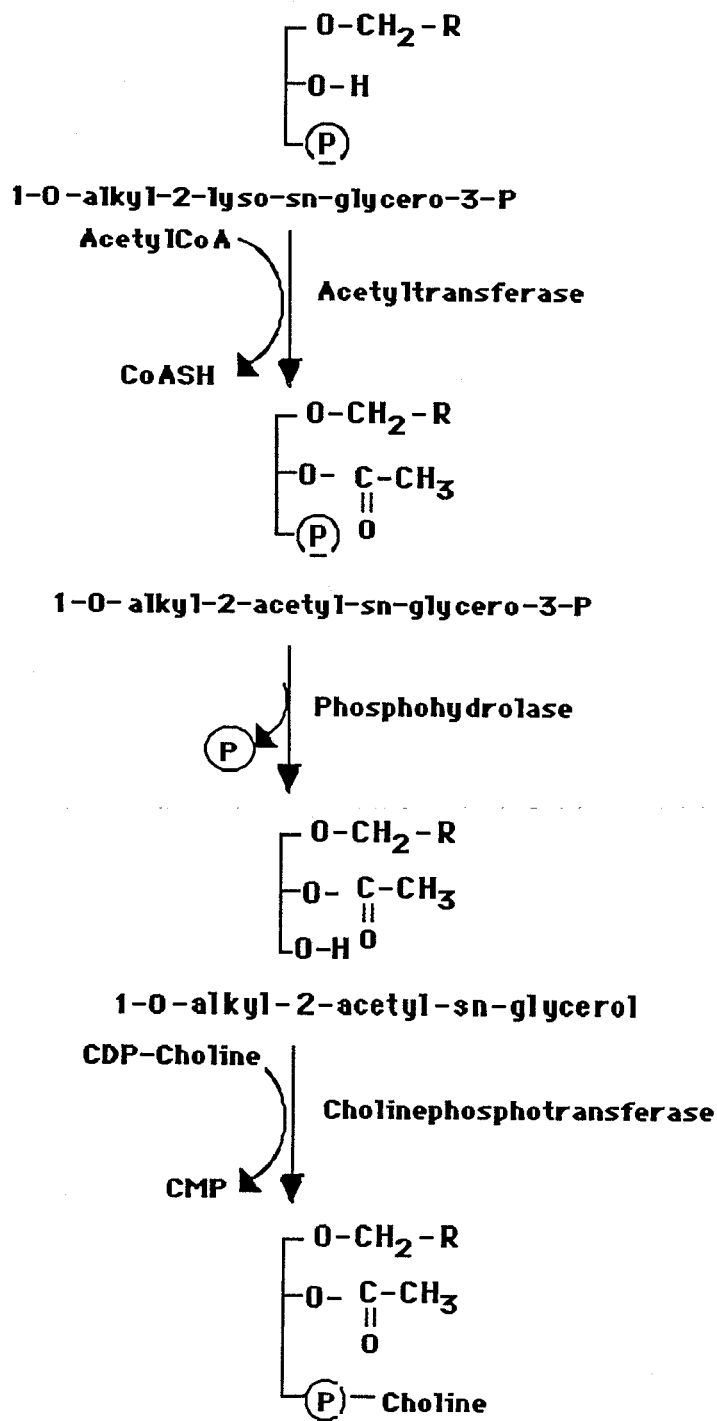
1.3 *PAF Biosynthesis*

In cells PAF can be synthesized via two known pathways; the *de novo* pathway and the remodelling pathway.

1.3.1 *De novo pathway of PAF biosynthesis*

In the *de novo* pathway 1-O-alkyl-2-lyso-sn-glycero-3-phosphate is converted to PAF in a stepwise sequence of reactions (figure 1.2). The first step in the *de novo* pathway involves the acetylation of an intermediate at the second carbon by a PAF specific acetyltransferase. A phosphohydrolase enzyme then dephosphorylates the compound at the third carbon. This is followed by the transfer of a phosphocholine from CDP-choline to 1-O-alkyl-2-acetyl-sn-glycerol by a CDP-choline phosphotransferase.

All the participating enzymes in the *de novo* pathway are located on the endoplasmic reticulum (6). Although the presence of CDP-choline phosphotransferase activity has been demonstrated in a number of tissues, thus far the physiological significance of the *de novo* pathway is unknown since upon cell stimulation by different agonists it appears that it is the remodelling pathway that is activated (6,7,8,9)



Platelet-activating Factor

Figure 1.2 *De novo* pathway of PAF biosynthesis, R= fatty acid (C₁₄-C₁₈).

1.3.2 *Remodelling pathway of PAF biosynthesis*

In the remodelling pathway the formation of PAF is initiated by the action of membrane bound phospholipase A₂ (PLA₂) (figure 1.3). The activation of PLA₂ appears to be calcium dependent and it is generally activated by agonists that also stimulate calcium mobilization (10). Once activated, PLA₂ deacylates membrane bound 1-alkyl-2-acyl-glycero-phosphocholine by cleavage at the 2(R) position thus releasing two products into the cytoplasm, 1-0-alkyl-sn-glycero-3-phosphocholine (lyso-PAF) and a free fatty acid. The free fatty acid most often released is arachidonic acid which serves as an important precursor for a number of inflammatory mediators via both the lipoxygenase and the cyclooxygenase pathway (11,12). The lyso-PAF that is formed is modified by a PAF specific acetyltransferase enzyme that attaches an acetyl group at the 2(R) position. Activation of acetyltransferase occurs within minutes of agonist stimulation and appears to be the rate limiting step in PAF formation (6,13,14,15). As seen with PLA₂, acetyltransferase activity correlates positively with calcium influx into the cell (16, 17).

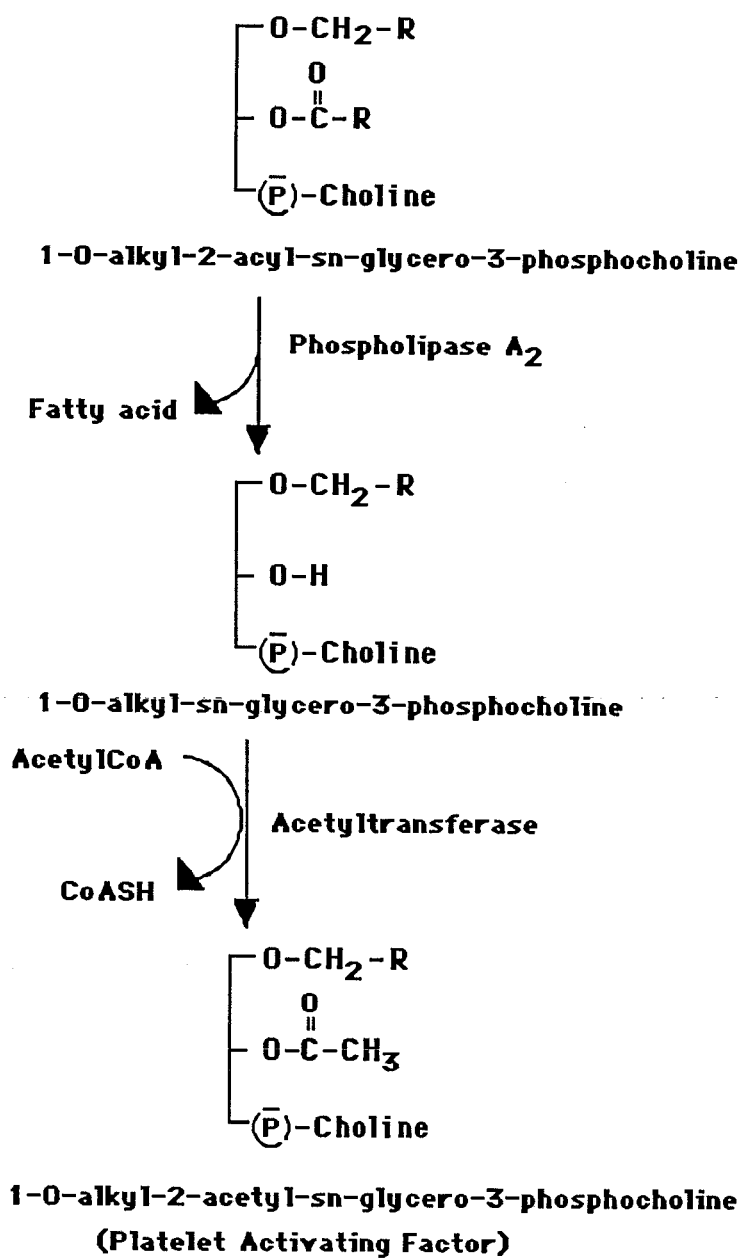


Figure 1.3 Remodelling pathway of PAF biosynthesis, R= fatty acid (C14-C18).

1.4 *PAF Inactivation*

PAF that is released from cells is metabolized and cleared from the blood very rapidly, occurring in approximately 30 seconds (18). Inactivation of PAF occurs in a two step process (figure 1.4). The first step in PAF degradation involves the cleavage of the acetyl group at the 2(R) position by an acetylhydrolase enzyme that is highly specific for phospholipids with short acyl chains at the sn-2 position. This results in lyso-PAF which is cytotoxic to the cell, having both lytic and detergent properties and therefore must be eliminated (19). Elimination is accomplished by an acylation reaction in which an acyltransferase converts the lyso-PAF formed to alkylacylglycerophosphocholine by the introduction of a long chain fatty acid onto lyso-PAF at the second carbon. It appears that phosphatidylcholine is the main source of arachidonic acid used to acylate lyso-PAF (20). The final step in the process of PAF inactivation is incorporation of the end product, alkylacylglycerophosphocholine into the cell membrane (21).

1.4.1 *Acetylhydrolase*

Acetylhydrolase is found in a variety of cells and tissues. It appears that both the intracellular and plasma forms of acetylhydrolase have identical substrate specificities but differ in molecular weight and do not cross react immunologically (18,22). In human neutrophils, acetylhydrolase appears to be a cytosolic enzyme (23). In human plasma 70% of acetylhydrolase is found tightly associated with a low density lipoprotein and the remainder is complexed with a high density lipoprotein. While both lipoproteins contain the same enzyme, it appears that the acetylhydrolase found with the low density form is more efficient at hydrolyzing PAF (24).

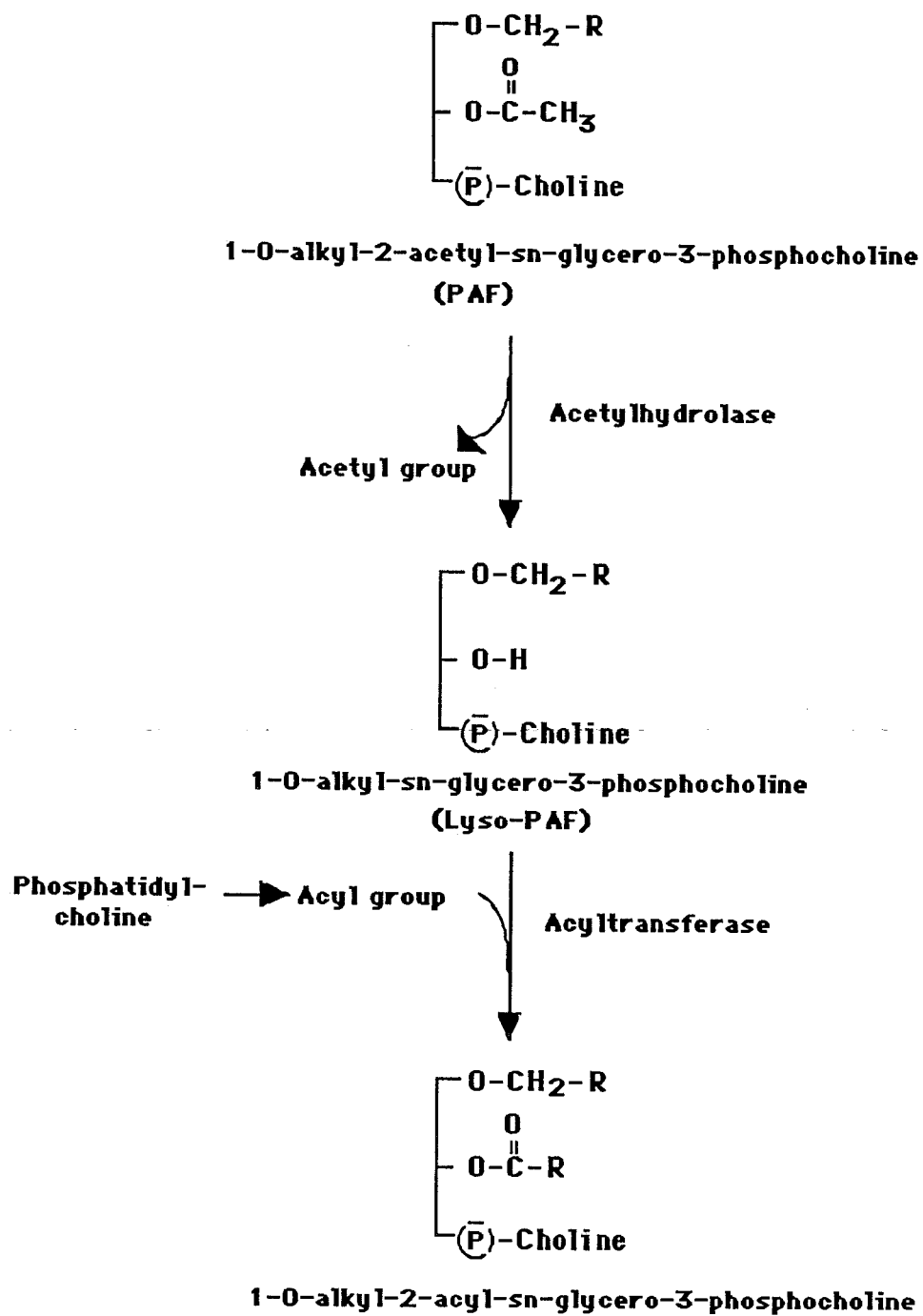


Figure 1.4 Inactivation of PAF, R= fatty acid (C14-C18).

1.5 Cellular Sources and Targets of PAF

1.5.1 Neutrophils

Inflammatory responses are characterized by the activation and infiltration of polymorphonuclear cells to the site of inflammation. When administered *in vivo* by a variety of routes, PAF causes transient neutropenia in both animals and humans with apparent sequestration in the pulmonary vasculature (25,26,27,28,29,30). *In vitro*, PAF is capable of causing neutrophil chemotaxis and enhanced neutrophil adhesiveness, aggregation, degranulation and superoxide anion generation (31,32). PAF can also prime neutrophils, thus resulting in a synergistic response when treated with other neutrophil agonists. Many of the other mediators of neutrophil activation can also prime neutrophils and not surprisingly simultaneously stimulate PAF formation (33,34). The list of mediators that can illicit PAF formation in neutrophils include: thrombin, collagen, Ca^{2+} ionophore A23187, f-methionyl-leucyl-phenylalanine and opsonized zymosan (35,36,37,38). In addition to PAF, many of these mediators also generate lyso-PAF. Therefore it is not unexpected to find that neutrophils contain comparatively high levels of the acetyltransferase enzyme which can readily convert synthesized lyso-PAF into PAF (8).

1.5.2 Eosinophils

Many inflammatory mediators have been shown to cause neutrophil chemotaxis but very few are able to cause eosinophil chemotaxis. *In vitro* essentially three important chemotactic substances are able to attract and activate eosinophils, leukotriene B_4 , eosinophil chemotactic factor and PAF (39). However, *in vivo* PAF appears to be the only mediator that selectively attracts human eosinophils (40). PAF is very effective in activating eosinophils to release basic proteins, leukotriene C_4 , oxygen radicals as well as more PAF (41,42,43,13). When eosinophils are stimulated with the Ca^{2+} ionophore A23187 they are capable of generating large amounts of PAF of which 30-50% is release from the cell (15).

1.5.3 Platelets

In vivo, PAF causes thrombocytopenia when administered intravenously and this is associated with the pulmonary sequestration of platelets (25,44,45). *In vitro*, PAF causes the aggregation of platelets from a number of animal species including, human, rabbit, guinea pig and dog and is the most potent inducer of platelet activation known (46,47). Rabbit platelets are extremely sensitive to PAF and will aggregate in response to concentrations as low as 0.1 nM (3,48). In platelets, PAF induces secretion of a number of endogenous mediators such as thromboxane A₂, platelet factor 4, ADP, serotonin and histamine. In addition, human platelet aggregation induced by some of these endogenous mediators can be potentiated by prior treatment with PAF through the upregulation of platelet fibrinogen receptors (46). The formation of PAF by platelets seems to be both species and agonist dependent. Chignard *et al.* (49) first demonstrated the formation of PAF in human and rabbit platelets in response to Ca²⁺ ionophore A23187 stimulation. Equivalent concentrations of Ca²⁺ ionophore A23187 evokes 2.99 ± 3.30 pmol PAF/5 X 10⁸ rabbit platelets as compared to 1.75 ± 1.40 pmol PAF/5 X 10⁸ human platelets (50). While PAF formation in platelets can also be induced by other platelet agonists such as thrombin and collagen (37, 38, 51), there also are several agonists recognized that do not induce PAF formation in rabbit platelets and this includes ADP, arachidonic acid and PAF itself (22, 37).

1.5.4 Endothelial cells

Endothelial cells are able to respond to as well as synthesize PAF. When stimulated with PAF, endothelial cells mobilize calcium, release prostaglandin I₂ and contract (52). When stimulated with other agonists, endothelial cells from a number of sources including bovine pulmonary and systemic vessels, rabbit thoracic aorta, as well as human pulmonary artery and umbilical vein have been shown to produce PAF (53,54,55). Human endothelial cell cultures are able to produce PAF when stimulated by thrombin, leukotriene C₄, leukotriene D₄, histamine, bradykinin, ATP, angiotensin II (54,56,57,58).

Essentially all of the PAF produced in endothelial cells remains associated with the cell (56,59,60). The PAF retained in endothelial cells is expressed on the surface of the cells and serves as an intercellular messenger (61). The interaction of neutrophils to the endothelial cells is an essential step in inflammation. Once neutrophils adhere to the endothelium they are free to migrate out of the vasculature towards the site of inflammation. It was found that treating endothelial cells with agonists known to induce PAF synthesis results in changes to the cell surface of these cells that promote the binding of neutrophils (56,62). It was determined that this interaction required the presence of an active PAF receptor on the neutrophil. It was also demonstrated that treatment of agonist stimulated endothelial cells with PAF acetylhydrolase reduced the binding of neutrophils to the endothelial cells (63). The role of PAF as an intercellular messenger on endothelial has also been established with regards to other circulating inflammatory cells such as platelets (61).

1.6 Involvement of PAF in Human Disease

1.6.1 Asthma

Asthma is a respiratory disorder characterized by the narrowing of the airways, mucosal edema and the influx of inflammatory cells. The result is dyspnea, wheezing and cough. This disease affects approximately 3% of the North American population and despite efforts aimed at improved diagnosis and treatment its prevalence and severity appear to be increasing (64). Theories put forth on its etiology include: airway epithelial damage, inflammatory cell infiltration, neurologic, cholinergic and/or mechanical abnormalities.

The hallmark of human asthma is airway hyperresponsiveness as defined by the exaggerated narrowing of the airways in response to nonspecific stimuli (65). In the airways of asthmatics there is a characteristic infiltration of inflammatory cells which include, eosinophils, mast cells and neutrophils (66,67,68). There is a close relationship between eosinophil count in the lungs and bronchial hyperresponsiveness (69). When stimulated, each of these cell types are a rich source of a

number of chemical mediators, each of which is capable of causing inflammation and bronchoconstriction. However, while many mediators are capable of causing epithelial damage and inflammation, PAF alone has been demonstrated to induce bronchial hyperresponsiveness and eosinophil chemotaxis *in vivo* (70). PAF's involvement in asthma is supported by both *in vitro* and *in vivo* data.

In vitro, PAF is able to induce contraction of isolated smooth muscle tissue which includes lung strips from rabbits, rats and guinea pigs (71,72). *In vivo*, intravenous injection and aerosol administration of PAF results in bronchoconstriction and nonspecific bronchial reactivity in a number of experimental animals including guinea pigs (73), dogs (74), rabbits (75), sheep (76), monkeys (77,78) as well as in normal human subjects (26,27). In rabbits and baboons PAF will cause thrombocytopenia, neutropenia and basopenia (79). In guinea pig airways PAF induced hyperreactivity is accompanied by the pulmonary accumulation of neutrophils, eosinophils and platelets and although a direct link to platelets has not been established, PAF induced airway hyperreactivity will not occur in some animal species when pretreated with antiplatelet antiserum (73,80). In humans, it was demonstrated by Cuss (26,27) that when normal subjects inhaled PAF they experienced bronchoconstriction and increased reactivity to methacholine for as long as 3 weeks after PAF challenge. Also in support of PAF's involvement in asthma is that it has been detected in the lavage fluid of asthmatic hyperreactives (81) and in the blood of allergic asthmatics following allergen-induced bronchoconstriction (82). But perhaps one of the strongest lines of evidence is the effectiveness of PAF antagonists in blocking PAF induced responses. Bronchial hyperreactivity induced by PAF in guinea pig model can be prevented by pretreatment with the PAF antagonists BN 52021 and WEB 2086 (83,84,85,86). These two agents were also effective in inhibiting eosinophil and neutrophil infiltration in guinea pig lung induced both by PAF and antigen stimulation (87).

1.6.2 Septic shock

Septic or endotoxic shock is the feature of blood contamination by bacterial toxin. It is defined by the inadequate perfusion of vital organs,

with associated systemic hypotension, decreased peripheral vascular resistance, and decreased cardiac output, all of which lead to total circulatory collapse (88,89). The morbidity and mortality rates for septic shock have not improved in the past 20 years and remains the most common cause of death in intensive care units in the United States (88,90).

To combat the assault on the body caused by the infection of gram negative bacteria, various systems are activated in response to the infection and include the complement, coagulation, kinin and endorphin systems (91,92). Circulating cells such as platelets and leukocytes are also activated and release a number of inflammatory mediators including PAF. Although a direct link to PAF is difficult to establish due to the complexities of this disorder, several lines of evidence show that PAF may play a strong role in the events leading to the hemodynamic instability observed during endotoxin induced shock. First, although PAF is not present in significant levels in control animals increased levels of PAF have been demonstrated in experimental models of anaphylaxis and endotoxemia. PAF has been detected in the serum from rabbits following antigen challenge (93), in the liver and spleen of sensitized mice following injection with IgG antibodies (94), in blood samples from rats following intravenous injection of *Salmonella enteritidis* (95) and in the peritoneal exudate and spleen of rats after intraperitoneal infusion of *Escherichia coli* (96).

Secondly, when administered systemically PAF is the single most potent endogenous agent known to produce a shock state (97). Striking similarities have been noted between effects of administered PAF and the hemodynamics of endotoxemia and anaphylaxis, including pulmonary hypertension, systemic hypotension, decreased cardiac output and enhanced vascular permeability. More importantly the reversal of shock states in experimental animals has been achieved with PAF inhibitors (96). The PAF receptor antagonist CV 3988 significantly reduced hypotension induced by both *Escherichia coli* and *Salmonella abortus* endotoxin infusion (98). Another PAF antagonist BN 52021 inhibited the hemodynamic effects of *Salmonella enteritidis* endotoxin on anesthetized rats and guinea pigs and significantly reduced mortality (99,100)

It seems that PAF's main effects may be mediated by the PAF generated release of secondary mediators. In the rat model, PAF is only able to cause the contraction of arterial strips in the presence of leukocytes or platelets. This action could be blocked with the thromboxane A2 antagonist ONO 3708. It would appear in this situation that the contraction of the arterial strips was a result of PAF-induced release of secondary mediator from the circulating cells (101).

1.6.3 *Kidney*

It is reported that the kidney is a sensitive target for the development of inflammatory tissue injury. Various properties of PAF make it the ideal candidate for a role in various kidney disorders and PAF has been implicated in the pathogenesis of glomerular injury as well as in various aspects of renal physiology. Both the mesangial and interstitial cells of the rat kidney can produce PAF under different experimental conditions (102,103). Furthermore, the detection of PAF in human urine has raised the possibility that PAF may be generated by the kidney even under normal physiological conditions (104). PAF possibly has a direct effect on mesangial cells of the kidney by inducing the production of various intermediates or else PAF may act indirectly by causing the renal deposition of circulating immune complexes. *In vitro* the application of PAF onto mesangial cells isolated from rat glomeruli causes cell contraction and prostaglandin E₂, superoxide anion and hydrogen peroxide synthesis (105,106). PAF can also cause the release of mediators from human mesangial cells and from isolated rat and rabbit kidneys (107). Cell contraction of the mesangial cells induced by PAF may favor the deposition of immune complexes in the kidney which serves to illicit the further release of inflammatory mediators. PAF's enhancement of vascular permeability may also facilitate the deposition of circulating immune aggregates (108,109,110). In the rabbit, PAF was shown to be released during kidney hyperacute allograft rejection (111). Also in the rabbit model, short term intrarenal administration of PAF resulted in platelet and neutrophil aggregation and degranulation in the glomerular capillaries. This resulted in the loss of glomerular negative charges due to the release

of cationic proteins and also resulted in mild proteinuria which lasted for several hours (40). Infusion of PAF in the renal arteries of anesthetized dogs is associated with a profound decrease of renal blood flow, glomerular filtration rate, and urinary sodium excretion. All of these reactions can be inhibited by prior infusion of BN 52021 (112). PAF antagonists can also reduce proteinuria and decrease the histopathological glomerular lesions in models of nephrotoxic serum nephritis in both rats and rabbits (113).

1.6 *PAF Antagonists*

There are a large number of PAF antagonists commercially available. Generally they may be broadly classified into two groups, structurally related compounds and structurally unrelated compounds.

1.6.1 *Structurally related PAF antagonist*

The first structurally related PAF antagonist reported was CV 3988 (114). This antagonist is derived from the PAF framework with the following structural features added; position 1 incorporates a octadecyl carbamate, a methylether occupies position 2 and a tiazolium ethyl phosphate is in position 3. At doses higher than those required for inhibition of PAF receptor binding, CV 3988 was also found to be effective against arachidonic acid, ADP (115), collagen (116) and calcium ionophore A23187 (117) induced platelet aggregation. In the rat, CV 3988 can inhibit PAF-induced hypotension (114), thrombocytopenia (118), endotoxin-induced disseminated intravascular coagulation (119) and counteract blood pressure decrease in the hypertensive model (120). CV 3988 can also prevent PAF-induced death in the mouse (121). In a double-blind, placebo controlled study, healthy volunteers received an IV infusion of CV 3988, this resulted in reduced platelet sensitivity to PAF (122). At the dose range of 750-2000 µg/kg it was found that blood pressure, pulse rate and respiratory parameters were unaffected.

Further modifications of CV 3988's basic structure has given rise to CV 6209, which has a octadecyl carbamate group in place of the phosphate

group at position 1 (2) and to SRI 63-119, which has a CH₂CH₂ unit in place of the PO₃ group in position 3 (123). Both compounds are effective antagonists of PAF-induced aggregation of both rabbit and human platelets, with CV 6209 having the greater potency (124,125). CV 6209 is approximately 80 times more potent than CV 3988 both *in vivo* and *in vitro* but is poorly absorbed per orally (126).

1.6.2 Structurally unrelated PAF antagonists

1.6.2.1 Naturally occurring antagonist-Ginkgolides

A family of naturally occurring PAF antagonists includes the ginkgolides isolated from the Chinese tree *Ginkgo biloba*. The ginkgolides have been designated A, B, C, M and J, corresponding to the names BN 52020, BN 52021, BN 52022, BN 52023 and BN 52024. Of the series BN 52021 appears to be the most potent and is able to inhibit PAF binding to rabbit (127) and human washed platelets (128,129) as well as to human leukocytes (127). Unlike CV 3988, BN 52021 inhibits aggregation of platelets only to PAF and not to ADP, collagen, arachidonic acid, thrombin or to calcium ionophore A23187 (115,127,129,130). BN 52021 is also effective in inhibiting PAF-induced internal Ca²⁺ increase in rabbit platelets (131,132), inhibiting PAF-induced aggregation and degranulation in human neutrophils (127), counteracting PAF and opsonized zymosan-induced leukotriene C₄ release in eosinophils (133) and inhibiting the effects of PAF on human endothelial cells (134).

In vivo, BN 52021 inhibits PAF and IgG-induced hypotension, hemoconcentration and extravasation in the rat model (135). In the guinea pig, BN 52021 antagonizes PAF and antigen-induced coronary vasoconstriction in the isolated heart (136,137) and antagonizes PAF-induced airway vascular permeability (138) and the release of thromboxane B₂, prostaglandin E₂ and leukotriene C₄ in isolated lungs (139).

1.6.2.2 Psychotropic agents-Triazolothienodiazepines

Triazolothienodiazepines are drugs classically employed as psychotropic agents. It was discovered that both alprazolam and triazolam

specifically inhibited PAF-induced human platelet aggregation (140) without affecting platelet response to ADP, thrombin, epinephrine, collagen, arachidonic acid or calcium ionophore A23187 (85,86). A separation of CNS and PAF antagonistic properties in the triazolothienodiazepine drugs was achieved with the synthesis of WEB 2086 (141). WEB 2086 selectively inhibits PAF-induced aggregation of human platelets and rabbit neutrophils (84,85,142). Studies indicate that WEB 2086 inhibits PAF receptor binding by interacting with PAF at a common site on the PAF receptor (142).

WEB 2086 can also play a therapeutic role when administered *in vivo*. In the guinea pig WEB 2086 inhibits PAF-induced bronchoconstriction, hypotension, thrombocytopenia and neutropenia (84,85,143). In the rat, WEB 2086 also protects against PAF-induced hypotension as well as against gastric lesions (84,85,144).

1.7 *PAF and Signal Transduction*

1.7.1 *PAF receptor binding*

Binding of PAF to the cell surface receptor initiates a cascade of internal effector systems which results in cellular responses such as chemotaxis, secretion or shape change. The PAF receptor has been identified in a number of cell types from a wide variety of species. Included in the list of cells which have been shown to possess the PAF receptor are rabbit platelets (145,146,147), human platelets (146,148,149) porcine platelets (150), human polymorphonuclear neutrophils (151, 152), human lung tissue (153), guinea pig smooth muscle (145) and rat liver plasma membranes (154). See table 1 for a comparison of binding affinities and receptor numbers in a variety of cell types.

| CELL TYPE | receptors/cell B _{max} | Binding Affinity K _d (nM) | REFERENCE |
|---------------------------------|------------------------------------|--|-----------|
| PLATELETS | | | |
| Human | 1,399 | 37 | 148 |
| | 242 | 0.59 | 147 |
| | 438 | 0.15 | 146 |
| | 1983 | 1.58 | 155 |
| Rabbit | 19,386 | 0.9 | 155 |
| | 689 | 0.68 | 150 |
| Porcine | 281 | 0.29 | 150 |
| Rats | 0 | 0 | 155 |
| NEUTROPHILS | | | |
| Human | 5.2 X 10 ⁶ | 0.11 | 151 |
| | 1,100 | 0.2 | 23 |
| Guinea Pig | 1.6 X 10 ¹¹ | 7.6 | 145 |
| CELL LINES | | | |
| HL-60 Cells (Differentiated) | 5200 | 0.7 | 156 |
| P388D1 Murine Macrophages | 7872 | 0.08 | 157 |

Table 1 Receptor number and binding affinity in platelets, neutrophils, HL60 cells and P388D1 cells.

PAF binding is distinguished by a number of characteristics. One is that a high degree of nonspecific binding to cell membranes is usually seen. This is likely due to PAF's hydrophobic nature which allows it to easily penetrate biological membranes (158,159). Although the lipid nature of PAF causes a high degree of nonspecific interaction with the cell membrane, it is speculated that PAF's lipid nature is necessary for the molecule to enter the lipid bilayer for interaction with its receptor (159). This was supported by studies by Duronio *et al.* (150) where no detectable PAF binding in whole cells was observed at 4° C and fewer receptors in membranes at 4° C were detected than at room temperature. It was proposed that increased membrane fluidity at higher temperatures facilitate the interaction of PAF with its receptor.

A second characteristic of PAF binding is its reversibility in some cell types and not in others (147). In neutrophils PAF is readily metabolized within minutes of contact and therefore receptor binding can be considered irreversible (23). This makes estimates of binding affinity and receptor number difficult and probably accounts for the wide range in number reported by different research groups. In contrast, PAF binding in platelets is stable and reversible. In platelets, one hour after [³H]PAF binding more than 90% of PAF remained unmetabolized and can still be competed off the receptor by excess unlabelled PAF (147,151,160).

A third aspect of PAF receptor binding is its sensitivity to both monovalent and divalent cations. Sodium has an inhibitory effect on PAF binding while potassium, cesium, rubidium, magnesium, calcium and manganese have an enhancing effect (161).

1.7.2 Receptor independent internalization of PAF

Receptor independent internalization of PAF was first put forth by Homma (162) and then by Tokumura (163). Subsequently Bratton (164) proposed that cellular activation of neutrophils leads to physical changes in the plasma membrane that in turn leads to the nonspecific internalization of PAF by a mechanism of enhanced transmembrane flipping. It was shown that internalization of PAF was nonspecific with regard to structural features of the molecule and did not require metabolism of the molecule.

The contribution and significance of this nonspecific uptake as compared to the receptor mediated uptake of PAF by neutrophils is not known.

1.7.3 *G-Protein*

Guanine nucleotide binding proteins (G-protein) are a family of proteins which bind and hydrolyze GTP. These heterotrimeric proteins are located on the cytoplasmic surface of the cell membrane and form links between the extracellular receptor and their effector systems. Included in this family of G-proteins are G_i which is reported to inhibit adenylyl cyclase, G_s which stimulates adenylyl cyclase and G_p which stimulates phospholipase C (165). Unlike G_i and G_s , G_p has not been biochemically identified and its existence is based upon evidence that fluoroaluminate (AlF_4^- ; a direct activator of G-proteins) and various GTP analogues are able to activate phospholipase C in intact cells and in membrane preparations (166,167,168,169,170). Also in support of a link between G_p and phospholipase C is the loss of inositol and Ca^{2+} signalling in some pertussis toxin treated cells (171,172). There are several reports indicating that the PAF receptor is linked to G-proteins (23,161,173). PAF stimulation of GTPase activity was demonstrated by the breakdown products of [g - ^{32}P]GTPase in rabbit platelet membranes in a manner that was receptor dependent and required Na^+ (161). Houslay *et al.* (174) demonstrate that activity of both G_s and G_i can be blocked by the NAD-dependent ADP-ribosylation of the active subunit with cholera toxin and pertussis toxin, respectively. In the case of G_p , it appears that at least two forms exist, one that is sensitive to pertussis toxin and a form that is not, however both are insensitive to cholera toxin (165). Using these known inhibitors of GTPase activity, it was shown that in platelets, PAF receptors are coupled to a phosphoinositide-specific phospholipase C through a G_p , in a manner similar to thrombin. The G-protein is insensitive to cholera toxin and only slightly sensitive to pertussis toxin (175,176,177). In contrast to platelets, PAF binding to neutrophils is inhibited by pertussis toxin implying a link to G_i or perhaps to the pertussis toxin sensitive form of G_p . Pertussis toxin was found to inhibit PAF-mediated chemotaxis,

superoxide generation, aggregation and the release of lysozyme from human neutrophils. (178). Similarly to other G_i linked receptors, direct addition of GTP inhibits specific binding of PAF to neutrophils (179).

1.7.4 *Phosphatidylinositol turnover*

Receptor mediated phospholipase C activation results in the phosphodiester cleavage of phosphatidyl inositol-4,5-bisphosphate (PIP_2) which gives rise to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). This occurs within 5-10 sec of PAF challenge (180). It appears that in platelets, PAF may cause the hydrolysis of a common pool of PIP_2 that is shared by thrombin (181). Both products of PIP_2 breakdown lead to the activation of calcium-activated, phospholipid dependent protein kinase C (PKC). IP_3 induces intracellular calcium mobilization leading indirectly to the activation of PKC (182). Recently, an IP_3 receptor has been purified in rat cerebellar Purkinje cells and was determined to be a ligand gated Ca^{2+} channel that exists in a multimeric form. The receptor contains an IP_3 binding site and shares many common features with the Ca^{2+} channel found on skeletal muscle sarcoplasmic reticulum (183,184,185). DAG is the second product of PIP_2 breakdown and remains in the plasma membrane where it binds with and activates PKC by increasing the affinity of PKC for phosphatidylserine and calcium, therefore activation can occur with lower calcium concentrations (186). Alternatively, evidence shows that intracellular Ca^{2+} mobilized by IP_3 may prime PKC to activation by translocating PKC to the membrane where small amounts of DAG can then activate the enzyme. Siess and Lapetina (187) showed that increases in calcium concentration alone could promote the translocation of PKC from the cytosol to the membrane. When examined further, the role of DAG, IP_3 -induced Ca^{2+} release and activation of PKC by PAF becomes even more complex. Pelech *et al.* (188) provide evidence that shows PKC activation by PAF appears to operate independently of translocation and that PKC activation is also independent of Ca^{2+} and lipid stimulation.

1.7.5 *Protein kinase C*

PKC consists of a family of lipid and calcium dependent isozymes all of which phosphorylate serine or threonine residues. The enzyme also serves as a receptor for phorbol esters, a class of tumour promoter (189). The evidence for DAG and IP₃ activation of PKC following PAF receptor activation includes the phosphorylation of a 40-47 kDa protein that serves as a marker protein substrate for PKC activity (190,191). This protein undergoes phosphorylation when rabbit and human platelets are treated with PAF (187,192,193). Sphingosine, a PKC inhibitor is also able to block the PAF-induced phosphorylation (194).

Although it is difficult to assign a direct role to PKC following PAF activation, several putative target substrates have been identified both at the level of PAF synthesis and at the level of signal transduction and cellular responses. At the level of PAF synthesis, there are at least two phosphorylation/dephosphorylation events which regulate PAF production. Phosphorylation appears to both activate acetyltransferase and to inhibit PLA₂. PLA₂ inhibition occurs indirectly through the phosphorylation of inhibitory proteins called lipocortins (195,196). Whether cAMP dependent protein kinase A (PKA) or calcium dependent protein kinase C (PKC) is involved in these processes remains to be clarified since both have been implicated (21,195,197). At the level of cellular responses, PKC activation leads to platelet activation and secretion (191,198). In various cell types PKC activation leads to both the phosphorylation of response elements as well as a dampening of some cellular functions. These opposing responses are poorly understood. It is found that direct activators of PKC either inhibit or enhance the degranulating actions of PAF in human neutrophils depending upon the concentrations used. It was found that treatment of neutrophils with the tumor promoter at concentrations that inhibited PAF-induced responses also caused a down regulation of high affinity PAF receptors (199).

1.7.6 *Adenylyl cyclase*

The activation of biochemical pathways following PAF binding causes the inhibition of adenylyl cyclase. In human platelets PAF inhibited the

basal, prostaglandin E₁-stimulated and fluoride-stimulated adenylyl cyclase activities (200). In human peritoneal macrophages physiological doses of PAF stimulated cAMP formation, likely through the formation of arachidonic acid metabolites. But at higher PAF concentrations cAMP formation was inhibited in the macrophages as in platelets (201). In neutrophils (178) and possibly in platelets (161,202) the inhibition is linked to the GTPase activity of G_i but PAF may also decrease cAMP concentration by increasing cAMP phosphodiesterase activity (200,203). A role for PAF-induced reduction of cAMP may be to increase intracellular Ca²⁺. Decreased cAMP may impair the function of cAMP-dependent calcium pumps which sequester intracellular calcium (204) and would otherwise inhibit phospholipase C-induced-PKC activation.

1.7.7 *cAMP dependent protein kinase A*

Formation of cAMP activates cAMP dependent protein kinase A (PKA) which like PKC also targets serine and threonine residues. It appears that activation of the PKA can lead to a dampening of PAF-induced responses at many levels. Undem *et al* (205) found that modest increases in cAMP-dependent protein kinase activity in mouse PT-18 mast cells leads to the inhibition of PLA₂ cleavage of arachidonic acid resulting in an inhibition of PAF synthesis. PKA may also play a role in inhibiting PAF responses following receptor activation. The IP₃ receptor isolated from the rat cerebellar Purkinje cells appears to be inhibited by PKA phosphorylation (206).

1.7.8 *Ion channels*

The application of PAF to target cells induces the mobilization of calcium from internal stores via the IP₃ receptor and from external sources via calcium pumps. PAF strongly modifies cell Ca²⁺ concentration in thymocytes, macrophages and vascular smooth muscle at concentrations greater than 10⁻¹² M (207). It is estimated that release of Ca²⁺ from internal stores accounts for less than 20% of the increase in calcium observed after PAF treatment (208, 209). In thymocytes, macrophages, platelets and vascular smooth muscle it is demonstrated that

PAF activates a Ca^{2+} pump. The effects of PAF on calcium influx were antagonized by the PAF receptor antagonist BN 52021. Platelet activation by PAF causes the opening of a calcium channel which is maximal within 5 sec and then closes with a half life of 45 sec (210). Evidence for the close association of PAF receptor with a calcium channel comes from experiments using calcium channel blockers. Calcium channel blockers, diltiazem and verapamil are able to inhibit PAF-induced calcium-influx, platelet activation and PAF binding (211,212). However, it must be noted that calcium channel blockers inhibit calcium influx by binding to channel-associated proteins rather than by directly blocking the channel. The identity of the protein remains unknown. The structural association of this protein with the receptor and the calcium channel also remains unsolved.

Although PAF was shown not to directly affect Na^+ transport systems, the increase in free cytosolic calcium did stimulate Na^+/H^+ exchange which is one main regulator of cellular pH and opened Ca^{2+} dependent K^+ channels which are frequently activated during secretory and inflammatory processes (213,214,215,216).

1.8 *Receptor Purification*

Until recently PAF receptor characterization has progressed at an extremely slow pace. Virtually no information existed as to the PAF receptor's structure. Past attempts at receptor characterization has been hampered by the lack of a consistent procedure to solubilize the membrane protein in an active form. The first reported isolation of the PAF receptor was by Valone (217). Human platelet membranes were solubilized with 5% sodium dodecyl sulphate and loaded on a sepharose column of PAF-human serum albumin. Following sodium dodecyl sulphate polyacrylamide gel electrophoresis a single protein with an apparent molecular weight of 180,000 was reported. However the identity of the isolated protein was in question. A second attempt to isolate the PAF receptor in human platelet membranes was made. Triton solubilization was followed by DEAE cellulose, CM cellulose and Sephadex G-200 separation. A protein with a molecular weight of 160,000 was isolated (218). Chau *et al.* (219,220) then made two attempts at receptor purification. The first attempt used a

sucrose density gradient and sephacryl S-300. This isolated a protein of 220,000 Da. The second attempt used a photoreactive radioiodinated derivative of PAF and isolated a protein of 52,000 Da. However, this group solubilized a [^3H]PAF receptor complex, leaving the possibility that the procedure could not be used to solubilize a unbound receptor that is not protected from denaturation. The authors also expressed difficulty in displacing [^3H]PAF following solubilization.

Recently, a successful solubilization of the unbound PAF receptor was accomplished by our group (221). An active form of the receptor was solubilized from rabbit platelet membranes and was shown to bind PAF with a similar K_d to that in intact cells. The purification was likely incomplete as reflected by the high molecular weight of 350 kDa reported. Important information obtained from this study included that the PAF receptor was heat labile and sensitive to trypsin inactivation and it was therefore confirmed to be a protein. Prior to this it was speculated that since PAF is a lipid it could in fact be binding tightly to lipid molecules in the membrane (222). The failure of the solubilized protein to bind to various lectin columns gave evidence that the PAF receptor is not a common glycoprotein containing N-linked oligosacchrides.

In a separate study using a gene expression approach, Honda (223) cloned the PAF receptor from guinea pig lung. This was then followed by cloning of the PAF receptor from leukocytes (224). This is the first phospholipid agonist for which the receptor has been cloned. A cDNA library was constructed from size fragmented guinea pig lung poly(A⁺) RNA that elicited an electrophysiological response in oocytes. The cDNA was a 3020 nucleotide sequence with the longest open reading frame translating to 342 amino acids. The predicted molecular mass of the receptor was reported to be 39 kDa. From this work the PAF receptor is now known to be related to a family of G-protein coupled receptors with seven putative transmembrane domains. The cytoplasmic tail of the receptor contains four serine and five threonine residues that could be possible sites for PKC phosphorylation. There are also 2 tyrosine residues that could serve as targets for regulation by tyrosine kinases. This study did not further characterize the expressed protein.

Chapter 2

Objectives

A number of goals involving the elucidation of the PAF receptor and PAF receptor signalling systems were targeted. My first goal was to study the effect of forskolin, an agent known to elevate cAMP levels in cells, on the PAF receptor-effector systems. My second goal was to evaluate the specificity of a PAF analog, 1-O-hexadecyl-2-O-(methylcarbamyl)-sn-glycero-3-phosphocholine (MC-PAF), which has potential uses in receptor purification and antibody production.

2.1 The effects of forskolin-an activator of adenylyl cyclase on PAF binding in rabbit platelets.

It has been shown that the PAF signal transduction system can be down-regulated by a widespread number of agonists. PAF binding and GTPase activity can be desensitized with prior exposure to PAF (146, 148). This is termed homologous desensitization as opposed to heterologous desensitization which occurs when a different agonist desensitizes the receptor. The effects of cross signalling between transduction signalling mechanisms are very interesting. It has been shown that thrombin-treated platelets are desensitized to subsequent exposure to both PAF and thrombin however pretreatment with PAF has no effect on the thrombin signalling system. Also indicated in several reports is that agents that elevate cAMP levels can also inhibit PAF-induced responses. It was shown in rat Kupffer cells that forskolin and dibutyryl cAMP were able to down-regulate the PAF receptor. It was assumed that the effects were induced through elevations of cAMP levels (225). These mechanisms of homologous and heterologous PAF receptor desensitization are poorly understood.

We decided to explore the phenomenon of cAMP-induced PAF receptor desensitization seen by Chao *et al* in Kupffer cells (225). The first step was to determine if cAMP-induced down-regulation of the PAF receptor can also occur in rabbit platelet and if so by what mechanism. Chao *et al*. (225) and many other researchers have used forskolin, as a tool to elevate

cAMP levels in cell systems. Classically it has been thought that forskolin binds directly to and activates the catalytic subunit of almost all mammalian adenylyl cyclase (226). As a result a number of the conclusions on the role of cAMP in PAF receptor desensitization are based on results observed with forskolin, however there are also non-cAMP mediated effects of forskolin which are often overlooked. The effects can be distinguished using dideoxyforskolin, an analogue of forskolin which does not activate adenylyl cyclase and will therefore serve as our negative control. Our objective was to determine if the effects seen with forskolin were a result of cAMP-induced down regulation and if so by what mechanism. Using different inhibitors and effectors of the post signalling system we will explore possible causes of PAF receptor desensitization.

2.2 Evaluation of methyl-carbamyl PAF binding to the PAF receptor.

Our goal in this study was to evaluate a commercially available analogue of PAF modified at the second carbon by the addition of a methyl carbamyl group. There are two foreseeable problems with the use of PAF in antibody production and receptor purification. The first is the presence of acetylhydrolases that rapidly inactivate PAF and the second is the lack of a functional group for conjugation. The addition of a methyl-carbamyl group to the sn-2 position on the PAF molecule theoretically makes MC-PAF more resistant to degradation by acetylhydrolases which could otherwise interfere with attempts at receptor purification and antibody production. If functional, MC-PAF would also give a more stable structure to which active groups could be added to the first or third carbon. However, we have seen that past attempts at modifying PAF's native structure can drastically decrease its binding affinity to its receptor. We needed to insure that this form of PAF retains its specificity and high affinity binding to the PAF receptor. We chose to evaluate the potency of MC-PAF in rabbit platelets because of the availability of proven bioassays to test its activity. In addition, binding assays to rabbit platelets are much more reliable than those performed in other cell types such as neutrophils in which there is rapid uptake and metabolism of PAF. The *in vivo* cardiac

effects of MC-PAF will be evaluated in anaesthetised rats, a model which has already been used effectively to evaluate the actions of PAF (227).

Chapter 3

Materials and Methods

3.1 *Materials*

PAF, forskolin, 1,9-dideoxyforskolin, 8-(4-chlorophenylthio)-adenosine 3'-5'-cyclic monophosphate (CPT-cAMP), bovine serum albumin (BSA), protease inhibitors, Ficoll-Hypaque and 3-isobutyl-1-methyl-xanthine (IBMX) were all purchased from Sigma Chemical Company (St. Louis, MO). Staurosporin, and N-(2-aminoethyl)-5-chloronaphthalene-1-sulfonamide hydrochloride (A3) were purchased from Biomol Research Laboratories, Inc (Philadelphia, PA). GF/C glass microfiber filters were purchased from Whatman Inc. (Clifton NJ). Cellulose acetate filters (HAWP, 0.45 μ M were obtained from Millipore Corporation (Bedford, MA). [3 H]PAF (80 Ci/mmol) and 5-hydroxy[G- 3 H]tryptamine creatinine sulphate ([3 H]serotonin) (11 Ci/mmol) and cyclic AMP [3 H]radioassay kit were obtained from Amersham radiochemicals (Arlington Height, IL). Ecolume and Cytoscint scintillation fluid was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA). 1-O-hexadecyl-2-O-(methylcarbamyl)-sn-glycero-3-phosphocholine (MC-PAF) was obtained from Cayman Chemical Co. (Ann Arbor, MI). WEB 2086 was obtained from Boeringer Ingelheim (Indianapolis, IN).

3.2 *Purification of platelets.*

Blood from healthy rabbits was collected in citrate dextrose. The blood was then mixed 3:1 with tyrodes buffer (0.25 % gelatin, 137 mM NaCl, 27 mM KCl, 12 mM NaHCO₃, 1 mM MgCl₂, 5.5 mM D-glucose), containing 0.1 mM of EGTA, pH 6.5. The blood was spun at 1200 rpm for 10 min in a Beckman GPR centrifuge (Fullerton, CA). The platelet rich plasma was overlayed on two mls of Ficoll-Hypaque and centrifuged at 3000 rpm for 15 min. The platelet bands located at the interface between the Ficoll-Hypaque layer and plasma was collected and pooled. The platelets were resuspended back to its original volume in tyrodes with EGTA, pH 6.5 and centrifuged at 3000 rpm for 15 min. The platelet bands were collected and washed twice in solution 1 (10 mM Tris-HCl pH 7.5, 2 mM EDTA, 150

mM NaCl), pH 7.5, by centrifugation at 3000 rpm for 15 min. The supernatant was discarded and the platelet pellets were resuspended in the appropriate assay buffer and counted by a Coulter Counter (Vancouver General Hospital, Dept. Haematology). Contamination by other cells types (white blood cells and red blood cells) was determined to be less than 1%.

3.3 *Membrane preparation.*

Isolated platelets were suspended in solution 2 (10 mM Tris pH 7.5, 2 μ M EDTA, 5 mM $MgCl_2$) containing 20 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor, 1 μ g/ml pepstatin and 20 μ g/ml PMSF and then stored at -80° C until membranes were prepared. Cells were thawed and sonicated on ice for 30 s at a 50% power setting on a Microson Ultrasonic Cell Disrupter (Heat Systems-Ultrasonics, Model HS-MS50, Farmingdale, NY). The membrane fraction was isolated on a cushion of 27% sucrose in 10 mM Tris/HCl, pH 7.5, by centrifugation at 65,000 x g for 20 min in Beckman TL-100 ultracentrifuge cooled to 4° C. Membranes were pooled and washed once in 10 mM Tris/HCl pH 7.5 and then resuspended in 1 ml of Tris/HCl pH 7.5. A 20 μ l aliquot was taken for protein determination by the method of Lowery *et al.* (228). BSA, $MgCl_2$ and KCl were then added in concentrations required for binding assays.

3.3 *Binding Assays*

The conditions for PAF binding to rabbit platelets and membranes were previously established in our lab (150). Prepared platelets were washed once with binding buffer (10 mM Tris-HCl, 0.25% BSA, 10 mM KCl and 5 mM $MgCl_2$) and then resuspended in binding buffer. 1×10^8 platelets or 10 μ g of platelet membranes were incubated in a total volume of 400 μ l of binding buffer for 2 min at room temperature in the presence of IBMX and other agents. Subsequently, [3H]PAF (final conc. 0.05 nM for whole rabbit platelets and 0.5 nM for rabbit membranes) and various concentrations of unlabelled PAF (0-75 nM for rabbit platelets and membranes) was added for a final volume of 500 μ l. Following a 20 min incubation at room temperature, the reaction was stopped by filtration on

Whatman GF/C glass fiber filters for the whole platelets and on cellulose acetate filters for platelet membranes. The filters were washed with 5 ml of binding buffer to remove excess unlabelled PAF, incubated in Cytosint scintillation fluid overnight and then counted in a liquid scintillation counter. The specific binding was obtained by subtracting the nonspecific binding in the presence of 75 nM unlabeled PAF from the total binding obtained.

For the experiment involving GTP-g-S, platelets were first treated with 10 µg/ml of saponin for 1 min. The saponin permeabilized platelets were then exposed to 200 µM of GTP-g-S and/or 50 µM forskolin or dideoxyforskolin for 2 min. [³H]PAF was then added and nonspecific binding was obtained by adding 75 nM unlabelled PAF.

3.4 *Platelet aggregation assay*

Prepared platelets were placed in tyrodes buffer with 1.3 mM calcium, pH 7.5. Aliquots of 4 X10⁸ platelets per ml were assayed for PAF-induced aggregation at 37° C using a Biodata aggregometer (Biodata, Hatboro, PA) which correlates platelet aggregation to changes in light transmission. PAF and MC-PAF or the drugs to be assayed were added directly to the platelets in a cuvette which contained a magnetic stir bar for rapid and homogeneous mixing. All PAF and MC-PAF concentrations were made in the above tyrodes buffer with 0.25% BSA. A blank of buffer alone was normalized as 100% transmission. A cuvette of platelet rich plasma was then calibrated as 0% transmission. Aggregation was measured as % increase in light transmission after the addition of PAF.

3.5 *cAMP assay*

Platelets in calcium containing tyrodes, pH 7.2 at 2×10^8 cells in 500 μ l were incubated with forskolin or various other agents for 5 min at room temperature. All samples also included 0.5 mM IBMX to inhibit phosphodiesterase activity. The reactions were stopped by the addition of 25 μ l of concentrated trichloroacetic acid. The platelets were pelleted for 5 min in a Beckman microfuge and the supernatant was subsequently removed for cAMP measurement. The level of cAMP in triplicate samples was measured by a cyclic AMP [3 H]radioassay kit.

3.6 [3 H]Serotonin incorporation into platelets

To incorporate [3 H]serotonin, isolated rabbit platelets in tyrodes with 0.1 mM EGTA, pH 7.0 were incubated at 37° C for two hours with 0.2 μ Ci/ml [3 H]serotonin. After the incubation period, the unincorporated [3 H]serotonin was removed by washing the platelets three times with tyrode's buffer, with EGTA, pH 6.5. The platelets were reconstituted to 2×10^8 cells/ml in tyrode's buffer containing 0.14 mg/ml CaCl_2 , pH 7.2.

3.7 Serotonin release assay

Aliquots of 0.5 ml of [3 H]serotonin labelled platelets were incubated with various concentrations of either PAF or MC-PAF for 2 min at 37°C. The reaction was stopped by centrifugation of the incubation mixture at 15000 X g for 15 sec in a Beckman microfuge. The supernatant (0.25 ml) containing the released [3 H]serotonin was mixed with Ecolume scintillation fluid and counted in a liquid scintillation counter. For accurate comparisons all samples were assayed in triplicate on the same day after challenge with the same batch of labelled rabbit platelets.

3.8 Serum acetylhydrolase assay

To assess the susceptibility of PAF and MC-PAF to serum acetylhydrolases we incubated 20 μ g samples of PAF and MC-PAF in sera obtained from rabbit blood. To obtain the sera, rabbits blood was allowed to coagulate for 1 hour on ice and then centrifuged at 2000 X g for 10 min. Samples were then incubated at 37° C in 0.2 ml of serum for various time

periods. The reactions were stopped by the addition of 0.5 ml of ice cold methanol. Proteins were allowed to precipitate overnight and then water and chloroform were added for a final ratio of 1:1:1. The chloroform layer was collected and PAF and MC-PAF were extracted from other contaminants in the sera by HPLC.

3.9 *HPLC extraction of PAF*

Each sample was dried under nitrogen gas and then reconstituted in 100 μ l of chloroform/methanol (9:1). The samples were then injected into a 4.1 X 150 mm, 10 μ M silica gel column (Altech Scientific Co., Deerfield, IL). A solvent system of isopropanol/toluene/acetic acid/H₂O (93:110:15:15) was used to extract PAF at a flow rate of 1 ml/min. The column was calibrated daily using [³H]PAF to generate a standard curve and determine the fraction at which PAF was eluted (figure 3.1). Five fractions on either side of the radioactive peak were pooled and evaporated under nitrogen. The dried residues were reconstituted in 100 μ l of tyrodes buffer containing 0.25% BSA and assayed for its ability to induce serotonin release from labelled rabbit platelets.

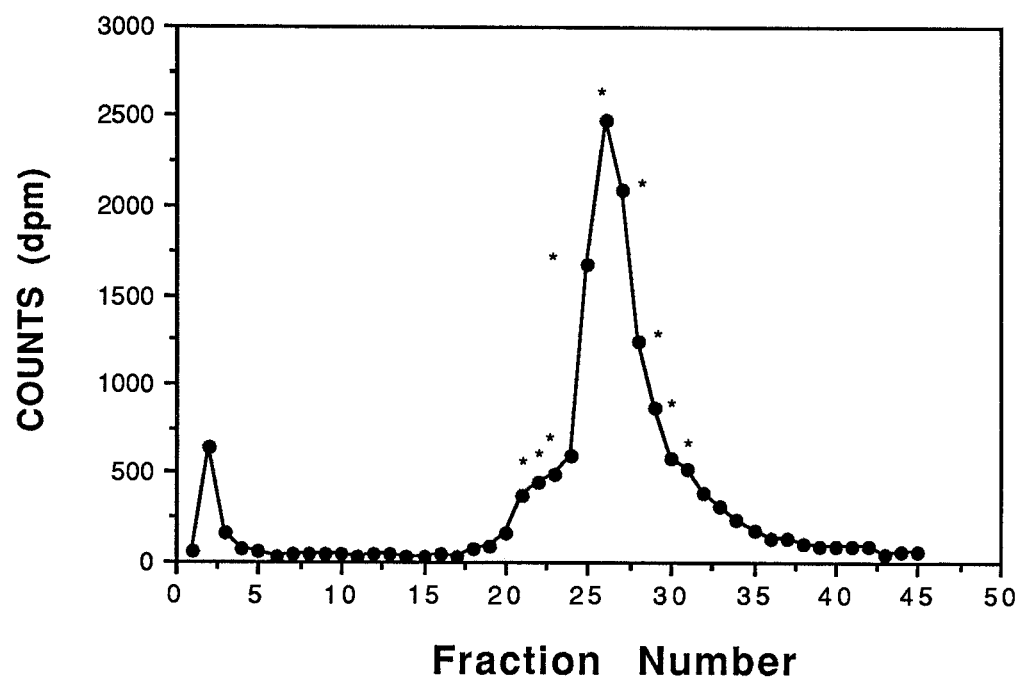


Figure 3.1 *Standard curve of HPLC extraction of PAF..*

Fractions marked by * indicates those collected and pooled for bioassay of PAF activity.

3.10 *Hemodynamics and cardiovascular effects of PAF and MC-PAF.*

Male Sprague-Dawley rats (200-300 g) were used in accordance with the guidelines of the University of British Columbia's Animal Care Committee. Rats (n=6 per group) were anesthetised with pentobarbitol (55 mg/kg i.p.) and their right jugular vein and left carotid artery were cannulated for drug administration and blood pressure monitoring, respectively. All animals were artificially ventilated via a tracheal cannula at a stroke volume of 10 ml/kg and a rate of 60 strokes/min to ensure adequate blood-gas levels (229). Body temperature was monitored by rectal thermometer and maintained between 36-37° C with a heating lamp.

The EKG signals were recorded via needle electrodes placed along the suspected anatomical axis (right atrium to apex) as determined by palpitation. A superior electrode was placed at the level of the right clavicle while the inferior electrode was placed on the left side of the thorax exemplifying Lead II configuration. EKG and blood pressure measurements were made directly from a Grass polygraph (model 7D) at a bandwidth of 0.1-40 Hz. Using a random block experimental design, animals were either given PAF or MC-PAF over a dose range of 0.5-20 µg/kg. Each dose was infused over 2 min and all recordings were made 3 min later, just prior to the addition of the next dose. The exposure time was chosen from preliminary studies at which pharmacological a steady state response to both PAF and MC-PAF occurred.

The *in vivo* data was analyzed for statistical significance by using the General Linear Model Analysis of Variance (GLM-ANOVA) followed by Duncan's multiple range test using the NCSS statistical package (230). All values are presented as the mean \pm s.d. A difference of $p < 0.05$ was considered significant.

Chapter 4

Results

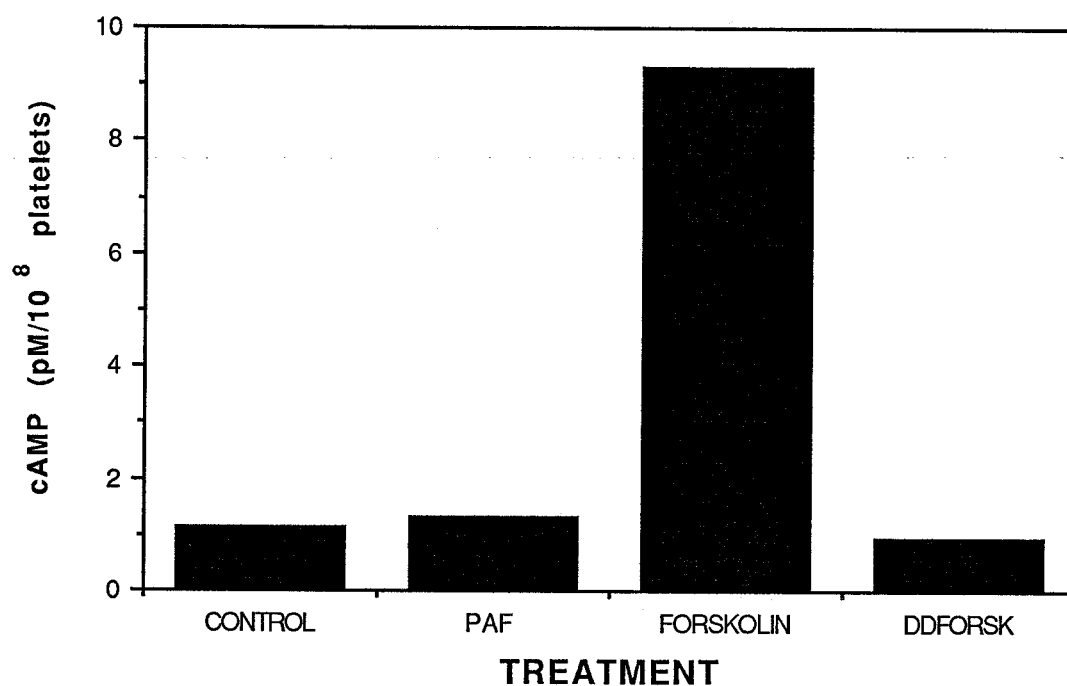
4.1 *Effects of Forskolin on PAF Binding*

4.1.1 *Measurement of cAMP in treated cells*

cAMP formation in rabbit platelets plays a major role in signalling mechanisms in the cell. We wanted to insure that the forskolin concentration of 50 μM used by Chao *et al.* (225) in Kupffer cells was adequate to stimulate cAMP formation in our cell system. When rabbit platelets were incubated with forskolin in the presence of IBMX for 2 min, approximately a 9-fold increase in cAMP was obtained. The level of cAMP in control platelets was approximately 1.1 pmol/ 10^8 platelets and this was increased to 9.2 pmol/ 10^8 platelets when treated with forskolin (Figure 4.1). As expected PAF and dideoxyforskolin had no effect on cAMP production in rabbit platelets.

Figure 4.1 *cAMP measurement in rabbit platelets.*

cAMP was measured in 10^8 platelets following 2 min treatments with PAF (2 nM), forskolin (50 μ M) and dideoxyforskolin (50 μ M). The results are the mean of triplicate determinations, and are representative of three experiments.



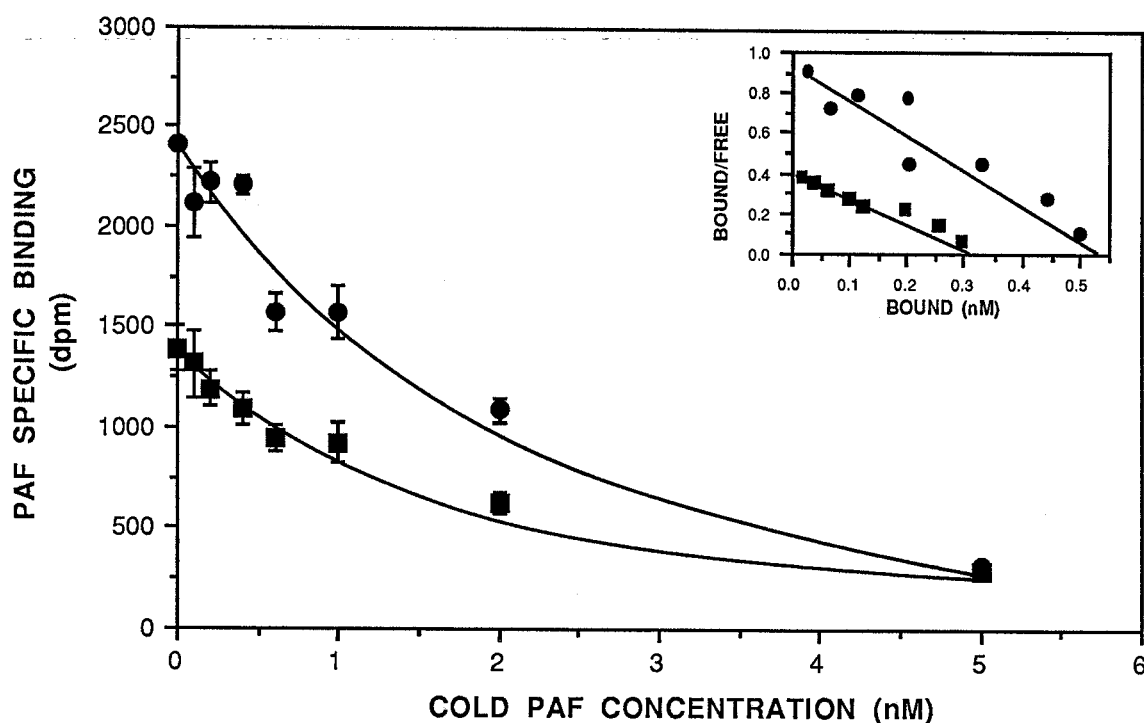
4.1.2 *PAF binding to forskolin treated cells*

Platelets treated with forskolin for 2 min demonstrated a decrease of approximately 30-40% in PAF binding (figure 4.2). Scatchard plot analysis of the data generated from binding of [³H]PAF to receptors treated with forskolin is also shown in the figure 4.2. Analysis of the data revealed that the decrease in PAF receptor binding was a result of a decrease in PAF binding sites (B_{max}) rather than a change in binding affinity (K_d). The K_d s obtained for PAF binding in control and forskolin treated platelets was 0.84 ± 0.13 nM and 0.81 ± 0.18 nM ($p > 1.0$, $n=5$) respectively. The number of binding sites obtained for PAF on platelets for control and forskolin treated cells were 1238 ± 199 and 747 ± 196 receptors/platelet, respectively ($p < 0.005$, $n=5$). The results shown are the mean \pm standard deviations. All binding data was performed in triplicate and the results of the scatchard analysis was analyzed for statistical significance using the Student t-test.

Figure 4.2 *Specific binding of PAF to rabbit platelets*

Platelets were exposed to 50 μ M forskolin (■) for 2 min prior to PAF binding. Control platelets (●) were treated with equivalent amounts of binding buffer. Each point is the mean of triplicate determinations with error bars indicating the standard deviation. The data is representative of similar results obtained in five separate experiments.

Scatchard analysis of PAF binding to rabbit platelets. The data generated from PAF binding to treated platelets is presented in the inset figure as a Scatchard plot. K_d for control (●) and forskolin (■) was 0.84 ± 0.13 and 0.81 ± 0.18 respectively. B_{max} for control and forskolin was 1238 ± 199 and 747 ± 196 receptors/platelet ($n=5$), respectively.



4.1.3 *CPT-cAMP, staurosporin and A3 treated platelets*

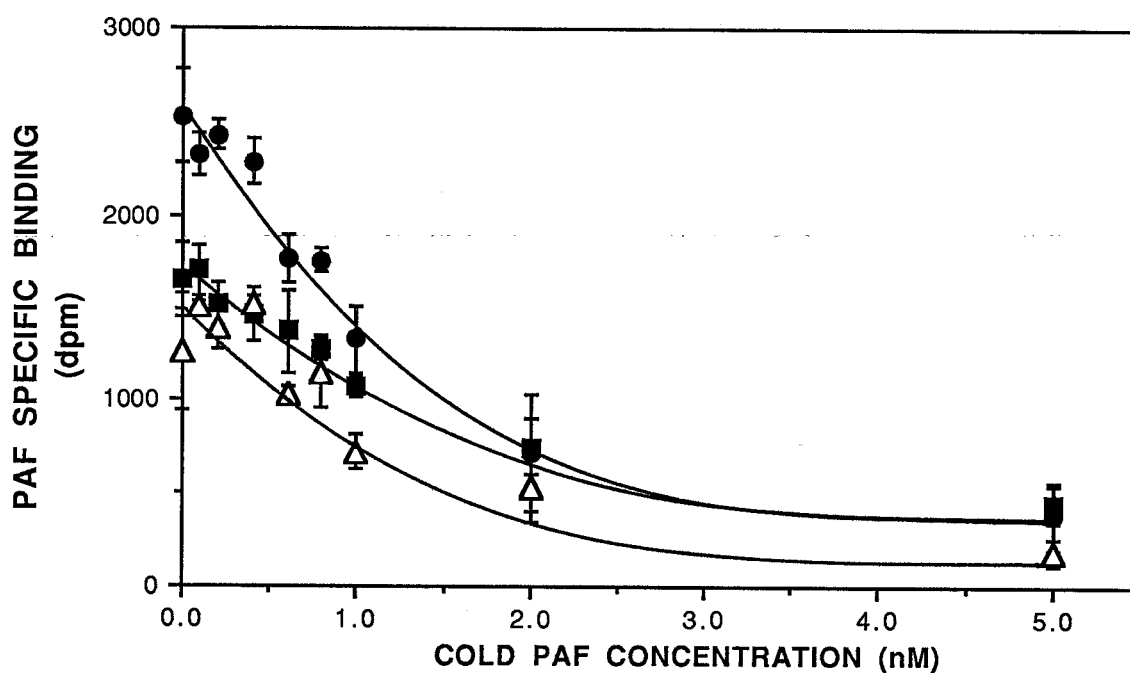
The effect of the membrane permeable cAMP analog, CPT-cAMP on PAF binding to rabbit platelets was investigated. Incubation of rabbit platelets with 10 nM and 100 nM of CPT-cAMP for 2 min did not induce any apparent changes in PAF binding (data not shown). This implied that cAMP was not the cause of the changes seen with forskolin treatment of platelets. We explored this further to determine if there was a possible activation of PKC or PKA in the action of forskolin. Treatment of platelets with the PKA inhibitor A3 (200 nM) (231) and the PKC-inhibitor staurosporin (1 μ M) prior to treatment with forskolin and dideoxyforskolin did not block the reduction in PAF binding (data not shown). This suggested that the action of forskolin was independent of PKA or PKC activation.

4.1.4 *PAF binding to dideoxyforskolin treated platelets*

The ineffectiveness of CPT-cAMP, staurosporin and A3 in inhibiting the effect of forskolin led us to believe that perhaps the effect of forskolin was independent of cAMP formation. Dideoxyforskolin does not activate adenylyl cyclase activity in cells and therefore can be used to distinguish cAMP dependent and independent responses. Treatment of platelets with dideoxyforskolin (50 μ M) also reduced PAF binding with the effect being approximately 10% greater than that seen with forskolin (figure 4.3).

Figure 4.3 *PAF binding to dideoxyforskolin treated platelets*

PAF binding to dideoxyforskolin (50 μ M) (Δ) treated platelets is compared to forskolin-treated platelets (\blacksquare) and untreated platelets (\bullet). Each point is the mean \pm standard deviation of triplicate determinations and is representative of 3 individual experiments.

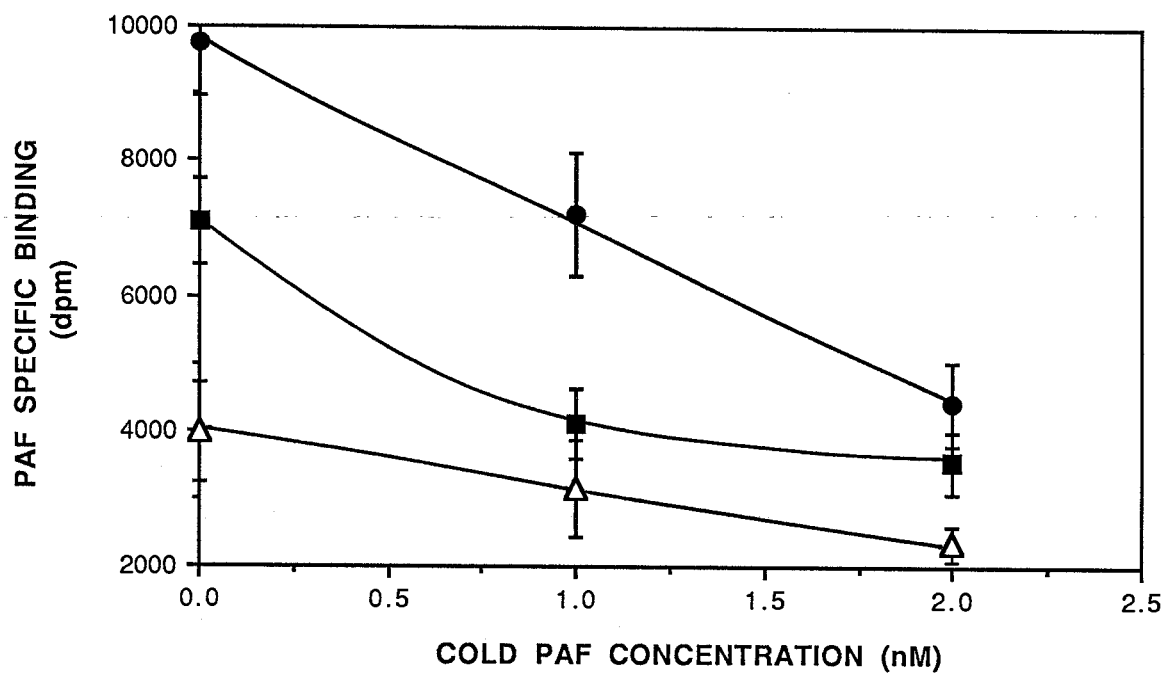


4.1.5 *Binding to forskolin and dideoxyforskolin treated membranes*

Experiments were performed with isolated rabbit platelet membranes to determine whether cellular activity was required for the actions of forskolin and dideoxyforskolin. Since previous experiments with staurosporin and A3 failed to reverse the effects of forskolin it was speculated that perhaps the effect of forskolin was due to a direct effect on a membrane bound component rather than through an activation of a signal transduction pathway. In these experiments, platelet membranes were treated with forskolin or dideoxyforskolin 2 min prior to the addition of PAF. As shown in figure 4.4, addition of forskolin or dideoxyforskolin to the purified platelet membranes reduced PAF binding. The changes seen with forskolin treatment of isolate platelet membranes was similar to those seen with forskolin treatment of whole platelets, suggesting the action of forskolin and dideoxyforskolin is on a membrane bound component and does not require an intact cell to be activated.

Figure 4.4 *PAF binding to treated membranes*

Membranes pretreated with forskolin (■) or dideoxyforskolin (△) are compared with untreated membranes (●). The experiment was repeated 3 times and each point shown is the mean \pm standard deviations (n=6) obtained in a representative experiment.

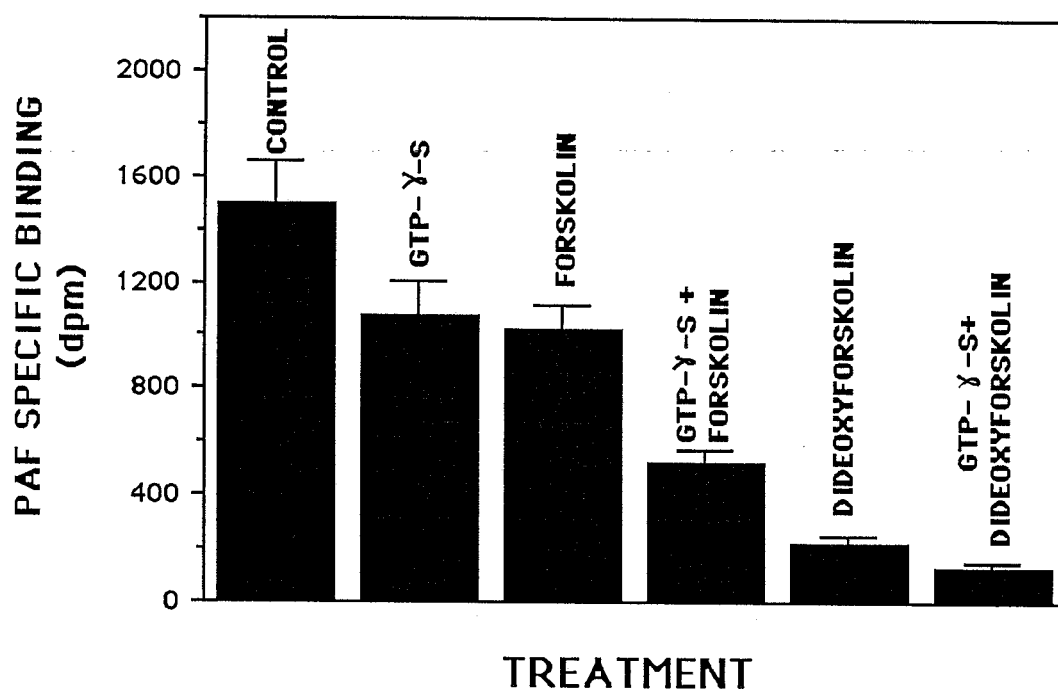


4.1.6 *Effect of GTP analog on forskolin and dideoxyforskolin treated platelets*

PAF receptor linkage to G-proteins has been established (see section 1.7.3). To explore the possibility that the effects observed with forskolin and dideoxyforskolin was a result of G-protein/receptor interruption, studies were performed with platelets treated with GTP-g-S in the presence or absence of forskolin or dideoxyforskolin. Binding of PAF to its receptors was found to be reduced by approximately 30% in forskolin treated cells (figure 4.5). When the two agents were added together PAF binding was reduced by approximately 70%. The results for dideoxyforskolin and GTP-g-S were also similar. These observations of an additive effect of GTP-g-S and forskolin/dideoxyforskolin suggested that forskolin and dideoxyforskolin were not acting on a G-protein to alter PAF binding.

Figure 4.5 *GTP-g-S treatment*

Saponin permeabilized rabbit platelets were treated for 2 min with 100 μ M GTP-g-S prior to the addition of forskolin or dideoxyforskolin. The results were compared to untreated platelets and platelets treated with GTP-g-S, forskolin or dideoxyforskolin alone. The results are the mean \pm standard deviations of triplicate determinations.

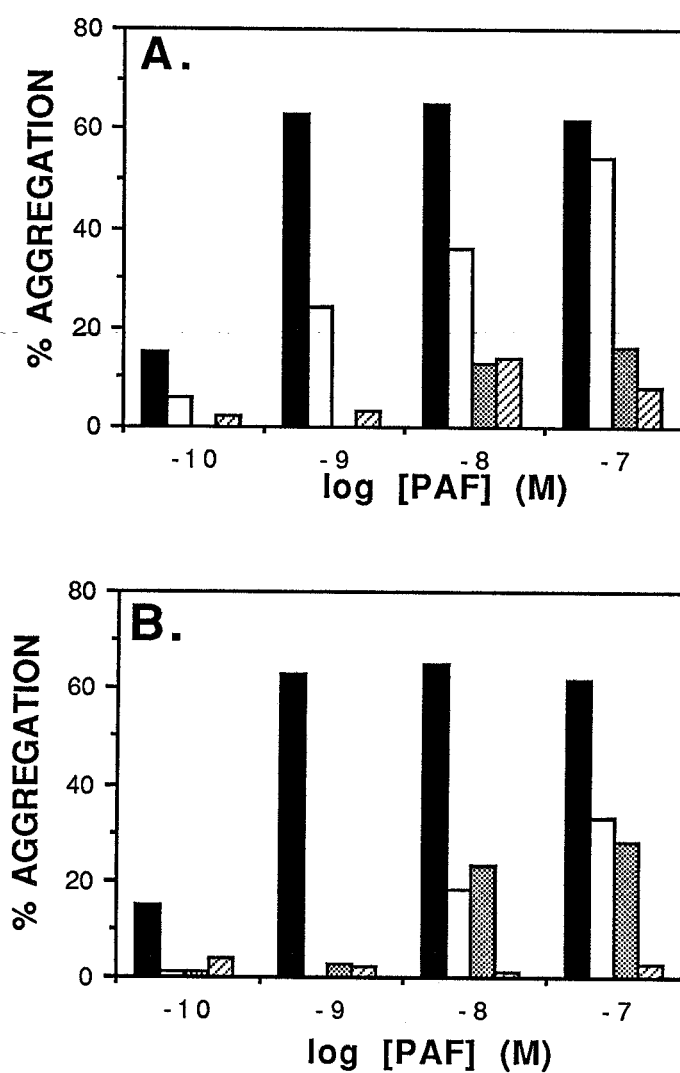


4.1.7. *Effect of forskolin and dideoxyforskolin on PAF -induced platelet aggregation*

To determine the physiological relevance of forskolin and dideoxyforskolin mediated changes in PAF receptor binding, platelet aggregation studies were performed. Washed rabbit platelets were exposed to either forskolin or dideoxyforskolin for 2 min prior to the addition of PAF. Aggregation in response to PAF was reduced significantly in platelets pretreated with either forskolin or dideoxyforskolin for 2 min prior to the addition of PAF (figure 4.6). Without forskolin or dideoxyforskolin treatment, platelets responded to PAF at less than nanomolar concentrations to induce aggregation, while platelets treated with forskolin or dideoxyforskolin resisted aggregation in response to PAF at much higher concentrations.

Figure 4.6 *PAF-induced platelet aggregation of forskolin and dideoxyforskolin treated platelets*

Rabbit platelets were incubated for 2 min with 0 μM (■), 0.5 μM (□), 5 μM (▤) and 50 μM (▨) forskolin (A) or dideoxyforskolin (B) prior to the addition of various concentrations of PAF. Aggregation was measured during a 1 min incubation.



4.2 *Evaluation of MC-PAF*

4.2.1 *PAF and MC-PAF-induced platelet aggregation*

Platelet aggregation is independent of cyclooxygenase products or ADP release and still is a method widely used to assess the potency of new PAF analogs (232,233). The popularity of platelet aggregation as a bioassay for PAF activity lies both in its simplicity and its relative sensitivity. Dose response curves were generated for both PAF and MC-PAF over the dose range of 0.01-100 nM. MC-PAF was able to mimic PAF in causing platelet aggregation but had a 2-4 fold lower potency than PAF (Figure 4.7). Full platelet aggregation was achieved with approximately 5 nM PAF as compared to approximately 10 nM for MC-PAF.

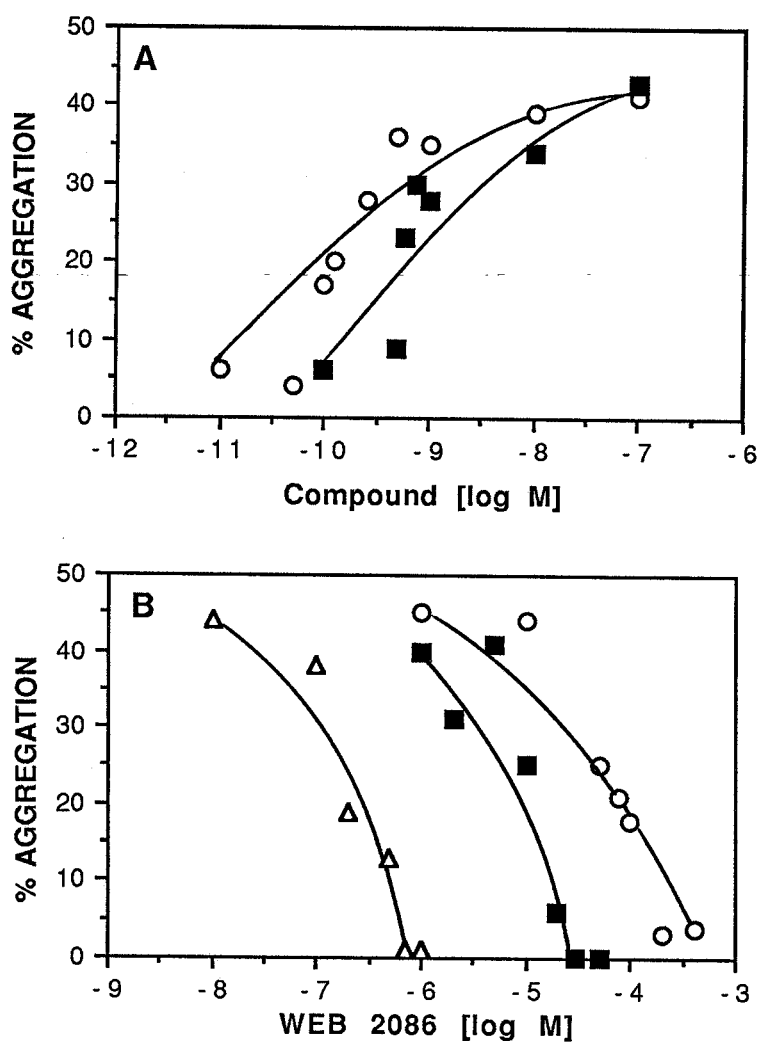
Next, the specificity of MC-PAF for the PAF receptor was assessed. We evaluated MC-PAF's ability to cause platelet aggregation in the presence of the PAF antagonist WEB 2086 which interacts with PAF at a common site on the receptor (142,84,85). Rabbit platelets were treated with various concentrations of WEB 2086 followed by 10^{-8} M MC-PAF. This test was repeated for the MC-PAF concentrations of 10^{-7} M and 10^{-6} M. WEB 2086 was able to competitively inhibit all concentrations of MC-PAF-induced platelet aggregation in a dose dependent manner.

Figure 4.7 A) *PAF and MCPAF-induced platelet aggregation.*

The potency of MC-PAF (■) was compared to PAF (○) in its ability to stimulate the platelet response to aggregation.

B) *Inhibition of MC-PAF-induced platelet aggregation by WEB 2086.*

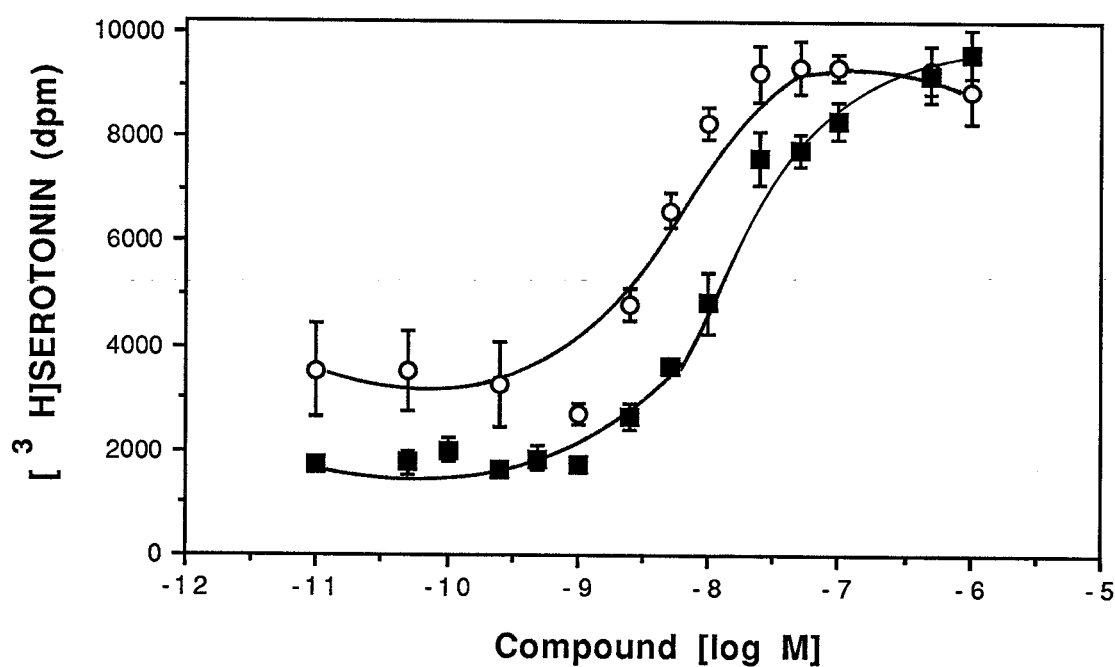
Platelets were first treated with the PAF receptor antagonist, WEB 2086 at various concentrations and then three different concentrations of MC-PAF was added: 10^{-8} (△), 10^{-7} M (■) and 10^{-6} M (○). In a dose dependent manner, WEB 2086 was able to block MC-PAF-induced platelet aggregation



4.2.2 *PAF and MC-PAF-induced serotonin release*

Serotonin is present in platelets at relatively high amounts and is taken up from the external media against concentration gradients via a membrane transport system coupled to sodium transport (234,235). All platelet serotonin is able to exchange freely with serotonin in the external media (235) and when platelets are activated by PAF and other agonists they release serotonin into the suspending media in dose dependent manner. This proven method of quantifying PAF activity was used to compare the in vitro physiological effects of MC-PAF with PAF (Figure 4.8). As with platelet aggregation the potency of PAF was about 2-fold greater than MC-PAF in causing serotonin release.

Figure 4.8 $[^3\text{H}]$ Serotonin release by PAF and MC-PAF in rabbit platelets. $[^3\text{H}]$ Serotonin labelled rabbit platelets were treated with increasing concentrations of PAF (○) or MC-PAF (■) ranging from 10^{-11} M to 10^{-6} M. The results are the mean \pm standard deviation of triplicate determinations and is representative of that obtained in two separate experiments.

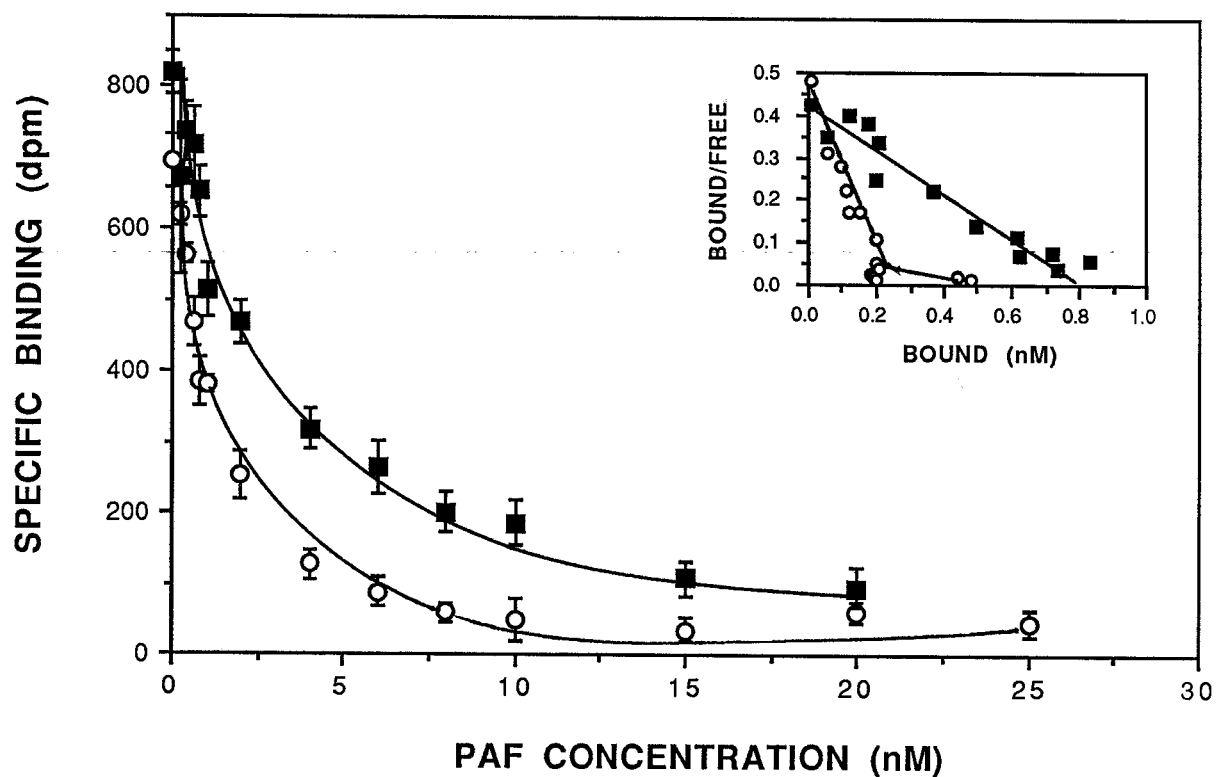


4.2.3 PAF and MC-PAF binding to rabbit platelets

The specificity of MC-PAF receptor binding was assessed in binding studies that were subsequently analyzed by Scatchard analysis to determine binding affinities and receptor numbers (figure 4.9). The K_d for PAF and MC-PAF were determined to be 0.62 ± 0.07 and 1.47 ± 0.30 nM, respectively (n=3). This approximately two fold difference was also reflected in the apparent receptor density values (B_{max}) of 750 ± 156 and 1931 ± 515 receptors/cell for PAF and MC-PAF, respectively (n=3). The PAF and MC-PAF binding studies were performed in triplicate in three separate experiments. The binding results confirmed that the difference in platelet aggregation and serotonin release recorded with PAF and MC-PAF are due to differences in binding at a receptor level.

Figure 4.9 *PAF and MC-PAF binding to rabbit platelets.*

Increasing concentrations of unlabelled PAF (○) and MC-PAF (■) were added to rabbit platelets in the presence of 0.05 nM of [³H]PAF. The points shown are the mean ± standard deviations of triplicate determinations and represent similar results obtained in three separate experiments. The specific binding data for PAF (○) and MC-PAF (■) shown in the inset.

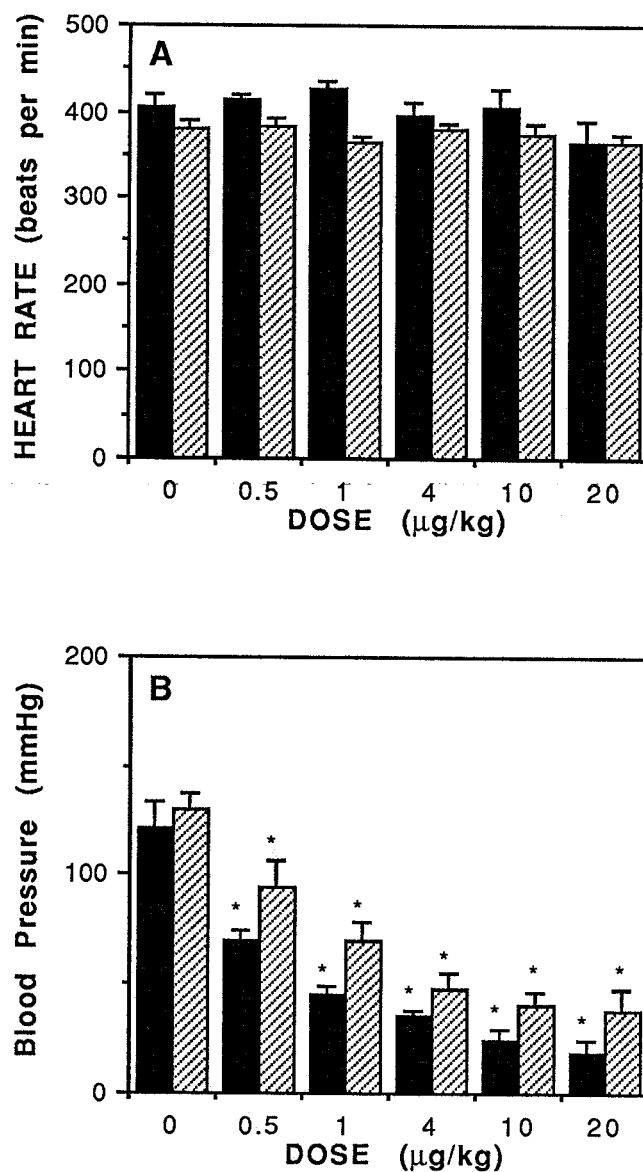


4.2.4 *Effect of PAF and MC-PAF on anaesthetised rats.*

In pentobarbitol anaesthetised rats, the cardiac and hemodynamic responses of MC-PAF were markedly similar to those of PAF. Neither of the two compounds significantly altered heart rate, even at a maximum dose of 20 µg/kg tested (Figure 4.10A). Both PAF and MC-PAF were able to reduce blood pressure in a dose-dependent manner (Figure 4.10B). The effect of both of these agents was instantaneous and persisted for at least 5 min after treatment. PAF and MC-PAF did not induce tachyphylaxis, even after the higher doses were administered. The dose at which blood pressure was reduced half maximally (EC₅₀) was approximately 0.5 µg/kg for PAF and 1.0 µg/kg for MC-PAF. A cumulative dose of 20 µg/kg of PAF reduced blood pressure to 18 ± 6 mm Hg while the same dose of MC-PAF reduced blood pressure to 37 ± 10 mm Hg. Studies of the EKG values showed that the P-R interval was slightly prolonged by PAF at higher doses but not by MC-PAF, while neither of the compounds altered the QRS or Q-T interval (results not shown).

Figure 4.11 *The cardiac and hemodynamic parameters of rats in response to PAF and MC-PAF.*

PAF (■) and MC-PAF (▨) from 1-20 $\mu\text{g/kg}$ were administered to anaesthetised rats. No significant changes in heart rate were observed (A). Both PAF and MC-PAF reduced blood pressure (B).

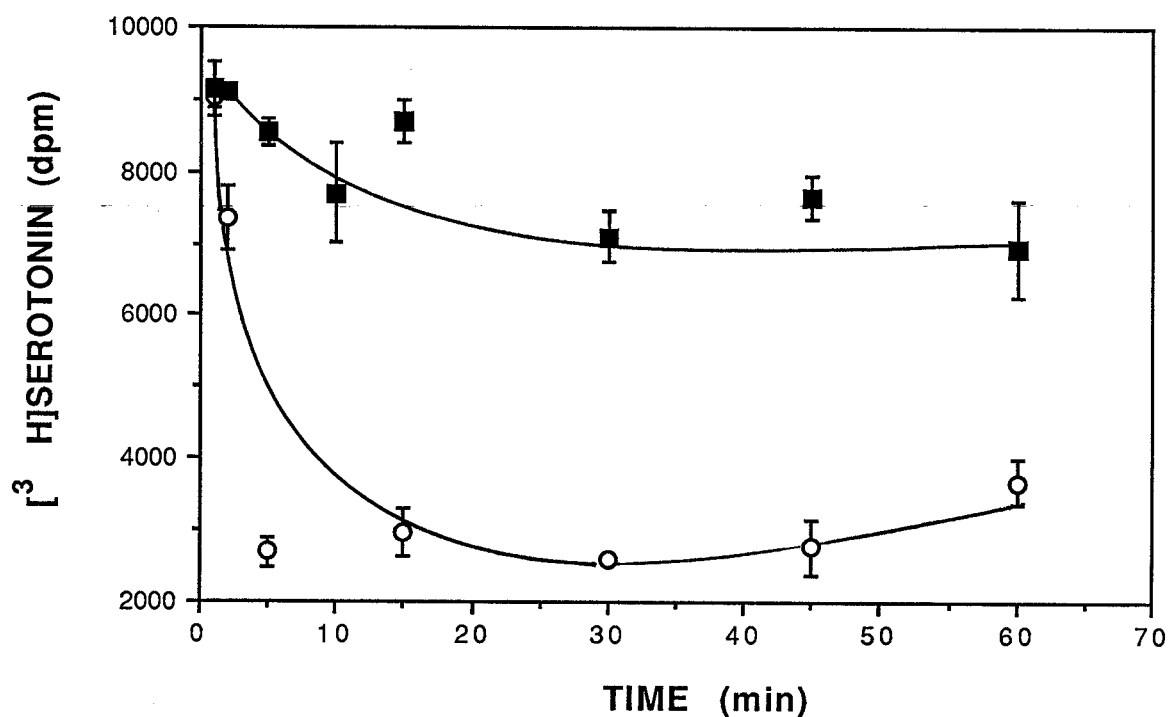


4.2.5 *Serum acetylhydrolase susceptibility of PAF and MC-PAF*

The conversion of PAF to its inactive metabolite, lyso-PAF occurs via the hydrolysis of the acetyl group at the sn-2 position by serum acetylhydrolases (see section 1.4.1). Since rabbits are often used as a vehicle for generating antibodies, PAF and MC-PAF inactivation in rabbit sera was assessed. PAF and MC-PAF were first incubated in rabbit sera for various time periods and then purified by HPLC. The resulting eluates were tested for their ability to induce [^3H]serotonin release from rabbit platelets (figure 4.12). There was approximately a 35% drop in PAF-induced serotonin release after only a two minute incubation in sera and this rapidly decreased at 5 minutes to a 75% drop in PAF stimulating activity. In comparison, MC-PAF resisted degradation by acetylhydrolases for up to 60 min and only experienced approximately 35% drop in activity.

Figure 4.12 *PAF and MC-PAF hydrolysis by serum acetylhydrolases.*

The susceptibility of PAF (○) and MC-PAF (■) to serum acetylhydrolases. After only a 5 min treatment PAF was rapidly degraded while MC-PAF was resistant to acetylhydrolase for at least one hour. Each point is the mean \pm standard deviation of triplicate determinations. The data represents results obtained in two experiments.



CHAPTER 5

DISCUSSION

5.1 *Effects of forskolin on PAF binding*

The results of the present study provide new insights into the action of forskolin on PAF binding. Forskolin was able to dramatically inhibit PAF binding to its receptor and subsequently block PAF-induced physiological responses. However, the results do not support the involvement of adenylyl cyclase in this process and suggest that other possible mechanisms must be explored when explaining forskolin's mode of action.

Although forskolin is best known as an activator of adenylyl cyclase activity, resulting in cAMP formation (226,236), it has been shown to have non-adenylyl cyclase activity in cells. Several reports have indicated that a number of forskolin-induced activities in various biological systems may be independent of adenylyl cyclase activation. Many of these activities are shared by dideoxyforskolin, an analog of forskolin which does not activate adenylyl cyclase (237). Dideoxyforskolin is therefore a useful tool in separating the cAMP dependent actions of forskolin from the cAMP independent actions. McHugh and McGee (238) demonstrated that both forskolin and dideoxyforskolin inhibited the nicotinic acetylcholine receptor mediated Rb^+ uptake in skeletal muscles that did not involve the activation of adenylyl cyclase or a cAMP dependent phosphorylation. Further work by Wagoner and Pallota (239) confirmed that forskolin-induced desensitization of the nicotinic acetylcholine receptor in skeletal muscles was mediated by a mechanism that did not involve the activation of

adenylyl cyclase or a cAMP-dependent phosphorylation. Another adenylyl cyclase independent action of forskolin includes the inhibition of glucose transport in human platelets, erythrocytes and adipocytes (240,241,242). These novel activities of forskolin are poorly understood.

Given the alternative actions of forskolin, we attempted to investigate whether the action of forskolin on PAF receptor binding was dependent on adenylyl cyclase. We were unable to replicate the effects of forskolin by direct addition of CPT-cAMP. Although Chao *et al.* (225) and others (243,244) were able to use dibutyryl cAMP (db-cAMP) to simulate the decrease in PAF binding shown with forskolin, we chose instead to use CPT-cAMP, bearing in mind that db-cAMP and forskolin have actions other than mimicking endogenous cAMP. It has been demonstrated that following uptake of db-cAMP, it is subjected to enzymatic degradation into monobutyryl cAMP and butyrate (245,246). Although monobutyryl cAMP is regarded as the active component, some effects of db-cAMP can be contributed to the action of butyrate. In one study, the inhibition of N2A cell growth, histone acetylation, thyroid hormone receptor down regulation and elevation of concentrations of histone H1 protein were all in part due to the presence of butyrate (247). To rule out the possible effects of butyrate in our system, we used CPT-cAMP which is known to be about 100 times more potent than cAMP in mimicking the effects of vasopressin in the mammalian kidneys. This is probably due to its greater resistance to phosphodiesterase activity and its greater permeability across cell membranes (248). Also Chao *et al.* (225) found that in order to achieve the effects seen with db-cAMP on PAF receptor binding, long term

treatment (24 hours) was necessary. This long incubation time injects some uncertainty into the results since others (249) have shown that even with 24 hour storage of platelets, responses to PAF and thrombin decline severely. The changes observed with stored platelets included decrease in receptor binding, aggregation and phosphoinositide turnover. We therefore chose to avoid long term incubations with any of the agents to prevent cellular degradation of the compound and to prevent possible physiological changes in our model that might be seen with long term storage. Using treatment times comparable to that of forskolin, we found CPT-cAMP to be ineffective in reducing PAF binding. Our aggregation data showed that the effect of forskolin was extremely rapid and thus we felt that treatment times with CPT-cAMP should be adequate. It was reasoned that if desensitization is a result of cAMP formation, the concentration CPT-cAMP used should also be adequate given that it is approximately 50, 000 times greater than the *in vivo* cAMP concentration measured in forskolin treated platelets.

After finding that CPT-cAMP was unable to mimic the results obtained with forskolin, we also found other lines of evidence that seemed to rule out the involvement of adenylyl cyclase and cAMP formation in the process of forskolin-induced alterations of PAF binding. This includes the ability of dideoxyforskolin to cause similar changes in PAF receptor binding. The ability of dideoxyforskolin to reduce PAF receptor binding conclusively eliminates the role of cAMP as the induced mediator. Chao *et al.* (225) did not evaluate the effect of dideoxyforskolin in their studies of cAMP desensitization of the PAF receptor pathway. Since forskolin was able to

affect the PAF biochemical pathways at the receptor level, the decreased PAF-induced arachidonic acid release and eicosanoid production observed by Chao *et al.* may also be attributed to a mechanism other than cAMP production. In addition, the positive effect that forskolin and dideoxyforskolin had on isolated platelet membranes demonstrates that these agents act at the cell membrane level and do not require the participation of intact cellular enzymatic systems. The results obtained with PKA and PKC inhibitors also indicate that although the PAF receptor possesses potential sites for protein phosphorylation (223), these two kinases do not participate in the reduction of PAF binding, unlike the mechanism of desensitization seen with the β -adrenergic receptor (250).

One possible explanation offered for the cAMP independent effects seen with the nicotinic receptor is that forskolin, an extremely lipophilic molecule is able to enter the plasma membrane, disrupt the lipid structure and interfere with normal channel activities in a similar way as a general anesthetic molecule. The observation of a slightly greater potency for dideoxyforskolin is consistent with the lipid perturbation theory, since dideoxyforskolin is a more lipophilic molecule than forskolin due to the lack of two hydroxy groups on the molecule (238). Another possibility is that perhaps forskolin and dideoxyforskolin were acting in a manner similar to that found for the glucose transporter where inhibition of activity was due to the direct binding of these agents to the transporter in rat adipocytes and human erythrocytes (251,252). It is possible that a hydrophobic interaction does exist and that binding of forskolin and dideoxyforskolin to specific sites may affect structures closely associated

with the PAF receptor. One possible site of action could be a guanine nucleotide binding protein (G-protein) to which the PAF receptor is coupled. Previous work has linked the PAF receptor to the G_i which in turn is known to play an important role in the control of adenylyl cyclase or to G_p which is linked to phospholipase C (see section 1.7.3). The latter results are supported by the work of Homma and Hanahan (244) which showed that db-cAMP pretreatment of rabbit platelets resulted in a decrease in the extent of PAF-induced GTPase activity. However in the present study, we propose that the action of forskolin and dideoxyforskolin is independent of G-protein involvement since GTP-g-S and forskolin had an additive effect on PAF binding.

In conclusion, the work presented in this study indicate that forskolin acts to reduce PAF binding to its receptor in a manner that does not seem to involve adenylyl cyclase, G-protein, PKA or PKC. Also we note that given the direct effect that forskolin has on PAF binding, caution must be used when it is employed as an agent to study the effect of cAMP on the PAF receptor and signal transduction pathways.

5.2 *Evaluation of MC-PAF*

The present work demonstrates that MC-PAF behaves in a manner representative of being a full PAF agonist. MC-PAF was evaluated both *in vitro* and *in vivo*. First, in all the tests of *in vitro* bioactivity MC-PAF was able to mimic the actions of PAF with only a two to five fold difference in activity. In receptor binding studies MC-PAF was shown to bind to the PAF receptor with only a two fold lower affinity than PAF.

Secondly, *in vivo* the cardiovascular changes induced by MC-PAF were very similar to PAF. It is noted however that MC-PAF does not reduce blood pressure to the same degree as PAF, even at the maximum dose of 20 µg/kg tested. The dose of MC-PAF that effectively reduces blood pressure half maximally, EC50, is approximately twice that of PAF. Thus the *in vivo* data also reflects the observations seen *in vitro* with rabbit platelets. As mentioned, tachycardia was not observed in our experimental model. This observation was first reported by Caillard *et al.* (253) however others report that tachycardia does not result from the severe systemic hypotension produced by PAF (254). Our results support the latter observations. The precise mechanism for PAF-mediated tachycardia under certain experimental conditions is not known.

Thirdly, MC-PAF was shown to be a relatively stable molecule as serum acetylhydrolases were not able to cleave the carbamyl moiety from the sn-2 position of the phospholipid molecule. The stability of MC-PAF should prove to be a valuable asset for investigations involving PAF and the PAF receptor. Potentially MC-PAF could be used for raising antibodies against PAF as well as in receptor purification.

Past attempts at PAF receptor solubilization have been hampered by the lack of a specific purification method for the PAF receptor. Although our research group has successfully solubilized an active form of the PAF receptor (221), the next step to be accomplished is a more precise purification of the receptor based on the solubilization procedures we have developed. Ideally this will be accomplished by the development of an affinity column in which PAF is immobilized on a solid support.

Theoretically, the solubilized PAF receptor could be then be applied to the column. Following multiple washes to rid the column of contaminating proteins the PAF receptor could then be eluted in a highly purified form. There are two foreseeable problems with our aims. The first problem is the presence of acetylhydrolases which can readily inactive the PAF bound on the affinity column into lyso-PAF a form unrecognizable by the PAF receptor. The second problem is that PAF lacks a functional group which can be used to immobilize it to a solid support. In this study, we have demonstrated that MC-PAF is resistant to acetylhydrolase activity. Therefore, by placing functional groups such as an amino or carboxyl moiety at the sn-1 position or by variations at the sn-3 position such as ethanolamine, monomethylethanolamine or dimethylethanolamine, it is possible to effectively conjugate the methylcarbamyl analog to a carrier for antibody production or to a solid support such as cyanogen bromide activated agarose for the purpose of receptor purification in an affinity column. Potentially, large amounts of PAF receptors could be solubilized and applied to the PAF affinity column. Elution of the column by appropriate buffers would result in large quantities of the purified receptor that would help tremendously in studies involving receptor characterization. Although the receptor has been cloned, information on the PAF receptor's association with other membrane bound proteins has yet to be determined. Also lacking is a crystalline form of the receptor. We believe that the information obtained from a solubilized form of the receptor would greatly aid in our understanding of how PAF is able to illicit so many diverse reactions in physiological systems. A more

complete understanding of the PAF receptor will also lead to therapies that can specifically diminish the actions of PAF in various pathological conditions.

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