CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

1-Hydroxyphenazine (1-HP) is a low molecular weight proinflammatory mediator derived from the *Pseudomonas aeruginosa* pigment, pyocyanine (Pyo). Pyocyanine and 1-HP are present in the airways of patients colonized with this microbial pathogen. Both pigments may contribute to microbial virulence and persistence by interfering with host defences (Ingram *et al.*, 1970; Wilson *et al.*, 1987). Cystic fibrosis is largely associated with chronic *Pseudomonas aeruginosa* infection. In the current study, I have attempted to identify the biochemical mechanisms which underlie the proinflammatory interactions of 1-HP with human neutrophils, and to identify potential anti-inflammatory strategies by which these potentially harmful effects can be neutralized.

1.2 CYSTIC FIBROSIS: The disease

Cystic fibrosis (CF) is one of the more common life-threatening inheritable disorders that was once thought to be found in people of European descent only. It has now been documented in South Africa in people of mixed ancestry, i.e. those of Koi/Hottentot/Malay/European descent in the Cape (Hill *et al.*, 1988). It has also been found in Asian people in America, and this is to say that its occurrence in any race is possible. Without treatment, most patients with this disease will die in infancy or early childhood. Otherwise, with early detection and treatment, sufferers reach adulthood. The disease is inherited as an autosomal recessive disorder of a gene located on chromosome number 7, band q31. The most common mutation at this site is a three-base pair deletion on the gene referred to as Δ508 (Kerem *et al.*, 1989; Riordan *et al.*, 1989), which encodes for a protein that serves as the chloride channel.

This protein is regulated by cyclic adenosine monophosphate (cAMP) and it is called the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Its absence or dysfunction in the apical membrane of epithelial cells is the cause of CF (Welsh *et al.*, 1995). Most patients with CF are diagnosed soon after birth by neonatal screening (Greene *et al.*, 1993). However, up to 5% of patients present with the disease as adults. Recently, the 46 year old mother of a CF patient was diagnosed with the disease as a result of molecular genetic analysis, illustrating the need to consider the diagnosis at any age in the close relatives of patients with CF (Gregory *et al.*, 1997).

The severity of the disease correlates with the degree to which the mutation affects the chloride transport. CF is a widespread disorder of the secretory processes of all exocrine glands, affecting both the mucus-secreting and the sweat glands. Thick abnormal mucopurulent secretions plug the pancreatic ducts and the bronchi resulting in most of the clinical features of this disorder. These include recurrent pulmonary infections leading to chronic lung disease, pancreatic insufficiency, steatorrhea, malnutrition, hepatic cirrhosis, intestinal obstruction and male infertility. Although multiple organs are affected in this disease, the most morbid and life-threatening pathology occurs in the lung (Engelhardt *et al.*, 1994). In the CF lung, host factors such as physicochemically altered pulmonary mucus, and possibly an increased number of receptors for *P. aeruginosa* on the surface of epithelial cells, impede the elimination of microbial pathogens. Bacterial pili, outer-membrane proteins, proteases, pigments such as pyocyanine, and mucoid exopolysaccharide contribute to the adherence and colonization during the initial phase of infection. Following this, *P. aeruginosa* uses a descending infection process to colonize and infect the lower airways. Given that opportunity, the immunoevasive arsenal of the bacteria then significantly potentiates invasion, resulting in chronic establishment of the pathogen (Buret *et al.*, 1993).

Electrolyte transport by the airway epithelium controls, in part, the quantity and composition of respiratory tract fluid. In this way, it helps to effect mucociliary clearance, this being the normal pulmonary defence mechanism, which removes inhaled particulate material from the airways. Chloride (Cl^-) is actively transported by the epithelium from the submucosa to the mucosal surface, thereby driving fluid secretions from the airways. However, in CF, it seems likely that the epithelia provide insufficient water to surface liquids in order to avoid inspissation of perciliary fluids and airway mucus (Fick, 1989). In CF, the primary defect is in the regulation of epithelial chloride transport. Normally, plasma membrane channels (chloride channels) transport chloride ions. The opening of these channels is mediated by agonist-induced increases in cyclic AMP which is then followed by the activation of cAMP-dependent protein kinase, resulting in the phosphorylation of the channel.

In sweat gland ducts, a defect in chloride transport leads to a decrease in the

reabsorption of sodium chloride (NaCl) from the lumen, resulting in an increase in sweat chloride. In the airway epithelium, the chloride channel defect in CF results in the loss of, or reduction of chloride secretions into the airways. Active sodium absorption is also increased in CF and both these ion changes increase water reabsorption from the lumen, lowering the water content of the mucus blanket coating mucosal cells. A schematic representation of the pathogenesis of the respiratory abnormalities in cystic fibrosis is shown in **Figure 1**.

The dehydration of the mucus layer leads to defective mucociliary action and the accumulation of hyperconcentrated, viscous secretions that obstruct the air passages and predispose to recurrent pulmonary infections. Mucociliary interaction and hence mucus transport in the airway is governed by ciliary motility, airway patency, mucus production, and airway fluid dynamics (Wanner, 1977). Obstructive lung disease and chronic bacterial infections lead to bronchiectasis and respiratory failure (Engelhardt *et al.*, 1994). Hypersecretion, recurrent and chronic pulmonary infection in CF, as well as accumulation of secretions in the airways, all lead to progressive deterioration of the lung and eventually death (Tizzano *et al.*, 1992).

Diagnosis of CF is based on at least two of the following: 1) a positive sweat chloride test which is above 60mEq/L in children and 80mEq/L in adults; 2) chronic obstructive disease of the airways; 3) exocrine pancreatic insufficiency; 4) molecular analysis; and 5) a positive family history of the disease. A positive sweat chloride test has been accepted as the primary signal for the diagnosis, but it is still prudent or safe to require at least one additional criterion.

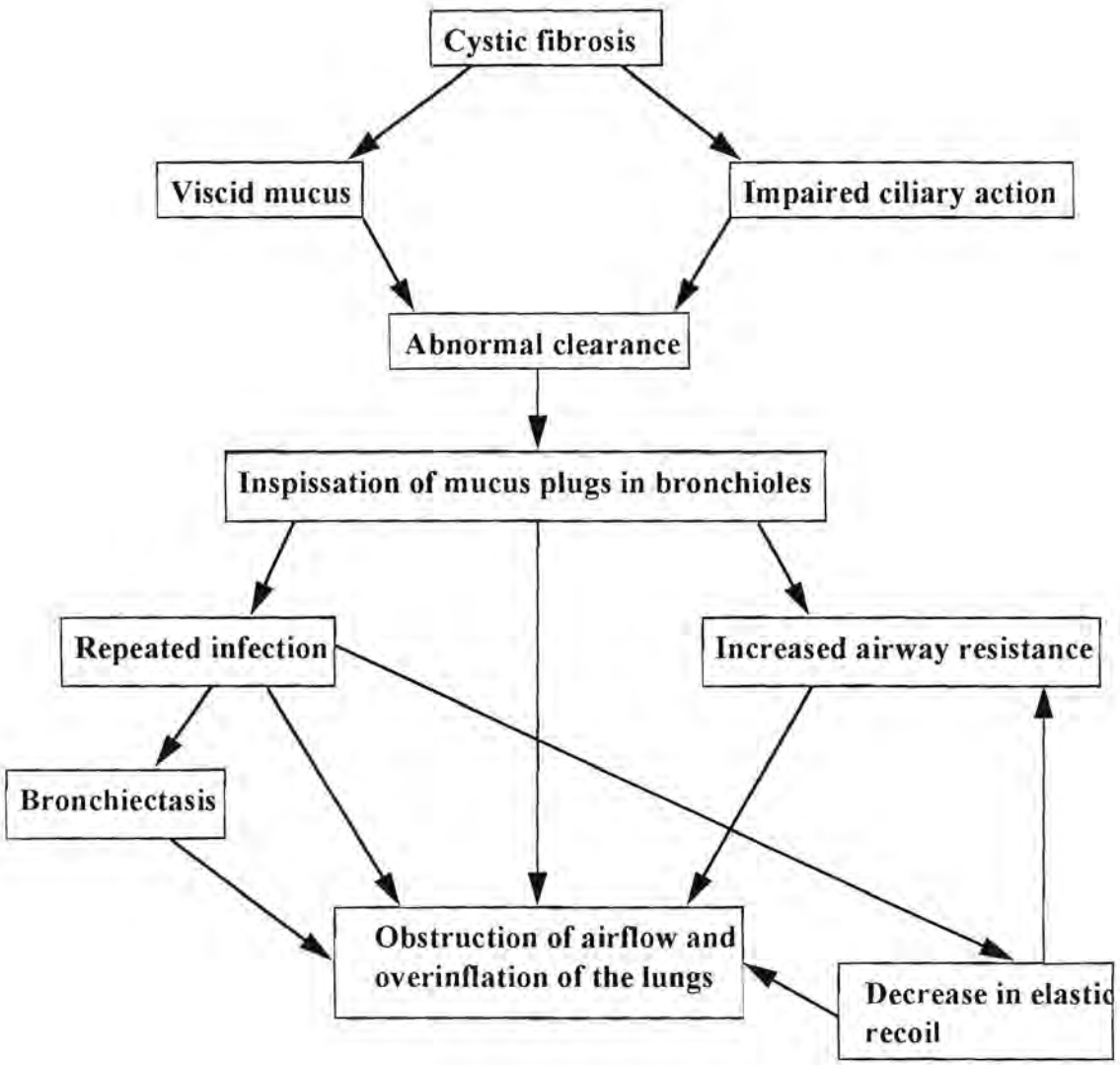


Figure 1: Schematic representation of the pathogenesis of respiratory abnormalities in cystic fibrosis

CF warrants special mention because after chronic *P. aeruginosa* endobronchial infection, this microbial pathogen persists in the airways of more than 90% of the patients (Koch *et al.*, 1993). Once established in the lung, *P. aeruginosa* is rarely completely eradicated regardless of the antimicrobial drug regimen used (Fick *et al.*, 1983; Davies, 1991). *P aeruginosa* is often the only pathogen recovered from the sputum or lung tissue obtained at thoractomy or autopsy, and clearly is the dominant organism to be reckoned with in this disease (Fick, 1989). By and large, present treatment regimens have increased the life-expectancies for many CF sufferers. Unfortunately, with *P aeruginosa* still remaining a scourge for these patients, development of even more effective antimicrobial agents is a priority.

2. PHAGOCYTES

Phagocytes are cells highly specialized for the performance of their primary functions. These are the phagocytosis and intracellular destruction of microorganisms. There are two principal types of phagocytes: (a) the intensely granulated, short-lived polymorphonuclear leucocytes, with neutrophils being the most abundant cell type and (b) the long-lived phagocytes of the mononuclear phagocyte system. This group includes the immobile fixed macrophages lining the lymph sinuses, Kupffer cells which line the blood sinuses of the liver, reticulum cells of the lymphatic tissues, monocytes in the circulation, alveolar macrophages in the lungs and microglia cells in the central nervous system (Anderson, 1991).

Neutrophils constitute the first line of cellular defence against infectious agents or non-self substances that penetrate the body's physical barriers (Smith, 1997). Their protective function in host defence is achieved through activation of their known chemotactic, phagocytic and degranulating properties (Andersson *et al.*, 1986). They are indiscriminate, aggressive, frontline phagocytes which transform molecular oxygen into a series of antimicrobial reactive oxidants when activated. This oxidant production is linked to the activation of a specialized enzyme system, NADPH oxidase, which is composed of membrane factors (cytochrome b_{558}, containing p22-phox and gp91-phox) and cytosolic factors (p47-phox, p67-phox, rac1 and rac2) (Umeki,1994; Bokoch, 1995).

The NADPH oxidase is a multicomponent, membrane-associated electron transport system, which when activated by various prooxidative stimuli, produces reactive oxidants which are antimicrobial. Assembly of the NADPH oxidase complex is completed by a selective coupling of activated rac1 and rac2 with p67-phox and p47-phox, respectively (Dusi *et al.*, 1995). The activation of NADPH oxidase involves the translocation of cytosolic factors, p47-phox and p67-phox to the plasma membrane, where they associate with cytochrome b_{558}, forming an electron transport chain responsible for the reduction of molecular oxygen (O_2) to O_2^-. During the course of the respiratory burst, a process associated with a dramatic increase in oxygen-uptake and metabolism in the neutrophil, reactive oxidants as well as granule proteases and antimicrobial polypeptides are released. These agents act cooperatively in the elimination of infectious microorganisms. It has become increasingly clear however, that the same beneficial armamentarium that is used by the neutrophil to defend the host against pathologic insults can also cause significant injury to the host tissues (Jackson and Cochrane, 1988; Anderson 1991).

In addition to the production of reactive oxygen species, the neutrophil releases myeloperoxidase and elastase, which alone, or in combination are potently bactericidal. Neutrophil myeloperoxidase, a granule-associated enzyme, in combination with H_2O_2, converts chloride to toxic hypochlorous acid/hypochlorite anion (HOCl) and chlorinated amine compounds. On a molecular basis, HOCl is 100 to 1000 times as effective as H_2O_2 against *Escherichia coli*. Unfortunately, it exerts a variety of unwanted effects, two of which are particularly dangerous. First, it can react with tissue cells, inducing depletion of ATP with consequent cell dysfunction and also cell necrosis (Dallegri *et al.*, 1988; Bernofsky, 1991). In part, these activities are mediated by fractions of HOCl which undergo transformation into chloramines (Bernofsky, 1991). Secondly, HOCl inactivates the essential protease inhibitor (α_1-antitrypsin) at the epithelial surface, but also inflicts damage on the epithelial cells. As a result, the airway epithelial barrier becomes more vulnerable to bacterial colonization and infection (Cantin and Woods, 1993; Lin *et al.*, 1997).

Recruitment or sequestration of neutrophils significantly increases the inflammatory

burden, which is exaggerated during airway infection. Neutrophils in the bronchial lumen are capable of releasing toxic oxygen radical species, including H_2O_2 and superoxide (O_2^-), as well as proteolytic enzymes (collagenase and elastase) that may cause goblet cell hyperplasia, impairment of mucociliary clearance, cleavage of immunoglobulins and complement, and increased mucus secretion. This may create a vicious cycle and exacerbate airway inflammation, as well as infection. Maximum stimulation of neutrophils due to "frustrated phagocytosis" leads to the production of large amounts of reactive oxygen species and proteolytic enzymes which overwhelm the innate anti-oxidative and anti-protease shields, thereby damaging vulnerable bystander proteins and cells (Zach, 1990).

Nitric oxide (NO) is produced in large quantities during host defence and immunologic reactions. It is generated from the amino acid L-arginine by nitric oxide synthase. This molecule serves as a biochemical messenger by binding to the heme of guanylate cyclase, and indirectly, as a cytotoxic agent. Although it is a radical, it is relatively stable, and like superoxide, it is not very reactive (Koppenol, 1998). Neutrophils undergo full activation with resultant generation of superoxide anions once recruited at sites of inflammation (Babior *et al.*, 1984). In turn, O^-_2 can react with NO, produced by extravasated neutrophils and tissue cells such as macrophages, fibroblasts and endothelial cells (Nathan, 1992). The reaction leads to the generation of peroxynitrite known to be a strong microbicidal and cytotoxic compound. However, anti-inflammatory properties have also been reported for NO. For example, inhalation of nitric oxide improves arterial oxygenation in high altitude pulmonary oedema. This beneficial effect may be related to its favourable action on the distribution of blood flow in the lungs (Scherrer *et al.*, 1996). Alternatively, or additionally, during acute inflammation, excess endogenously produced NO will inhibit NADPH oxidase in the membrane of neutrophils, thus limiting the availability of O^-_2, and providing defence against free radical-mediated injury (Rodenas *et al.*, 1998). The formation of peroxynitrite from NO can be prevented by lowering the concentration of O_2^-. Superoxide (O_2^-) can be lowered by superoxide dismutase (SOD) or SOD-mimics (Koppenol, 1998).

A variety of antioxidants are distributed in plasma and in tissues, including the lung and

the liver. They are thought to have the potential to protect against tissue damage and immune dysfunction mediated by phagocyte-derived reactive oxidants (Anderson *et al.*, 1990). While controlled release of reactive oxygen species is essential for normal phagocyte function (e.g bactericidal function), excessive generation of reactive oxygen species may be harmful, especially when it exceeds the antioxidant capacity (Fantone and Ward, 1982). Imbalances between the production of oxidants and their inactivation by the antioxidant defence system may contribute to the pathogenesis and resultant complications of CF. As long as there is an adequate amount of antioxidants present to exert efficient protection, the oxidant-antioxidant balance is maintained (Winkelhofer-Roob, 1994).

3. PROTEASES

3.1 Neutrophil elastase (NE)

The most dramatic change to occur in CF airways after colonization with *Pseudomonas aeruginosa* is the rapid recruitment of neutrophils from the bloodstream. These phagocytes become the most numerous cell type in the airways of most chronically infected CF patients and are a source of a large burden of proteases, particularly neutrophil elastase (NE). Proteases are proteins that function as enzymes and have the capacity to degrade other proteins by hydrolyzing peptide bonds. Some function within cells and others are released by the cells into the local mileu and are then capable of modifying the extracellular matrix components or the cells of the lung parenchyma. This is usually prevented in a regulated fashion by antiproteases at sites where there is a low level traffic of activated neutrophils in which elastase activity in the tissue is moderate.

NE has been implicated as being a factor that impairs local host defences in chronic *P. aeruginosa* lung infection in CF (Tosi *et al.*, 1990). When neutrophils are activated or lysed, e.g. during the process of phagocytosis, NE and other enzymes may escape from the phagolysosome and reach the extracellular space, where they may cause substantial damage to the surrounding lung tissue, inhibit critical functions, and inactivate molecules relevant to the integrity of the lung (Doring *et al.*, 1981).

Neutrophil elastase is a 29-kD glycoprotein with broad-spectrum proteolytic activity at neutral pH. It is produced in the bone marrow in the promyelocyte stage during the process of neutrophil differentiation. It is then stored in the primary (azurophilic) granules of the neutrophil. Physiologically, NE degrades nearly all structural proteins of the lung including elastin, collagen types I-IV, fibronectin, laminin and the protein components of the proteoglycans (Woods *et al.*, 1982; Azghani *et al.*, 1991). Studies *in vitro* indicated that cell surface fibronectin protected against adherence of gram-negative bacteria to epithelial cells. Woods *et al.*, (1982) showed that removal of fibronectin from epithelial surfaces promoted adherence of *P. aeruginosa*. It has also been shown that the absence of fibronectin on epithelial cell surfaces from patients with CF, and from those seriously ill patients with acute respiratory failure, correlated well with increased adherence of *P. aeruginosa* to buccal epithelial cells (Woods *et al.*, 1980; Woods *et al.*, 1981; Suter *et al.*, 1988). NE may also inactivate several components of the immune system, such as immunoglobulins, immune complexes, complement components and cell surface receptors on neutrophils, leaving them almost incapable of opsonization and elimination of bacterial pathogens (Berger *et al.*, 1986; Birrer *et al.*, 1994).

NE and oxidants have also been found in the pulmonary tissues of neonates and adults suffering from acute respiratory distress syndrome. They have also been demonstrated in various chronic pulmonary disorders of infective and non-infective origin. For example, lavage fluids from cigarette smokers contain active NE, which is not bound to α_1-PI, because the latter had been oxidized at the active site with resultant inactivation of anti-protease activity (Lee *et al.*, 1981; Cochrane *et al.*, 1983; Janoff *et al.*, 1993). Uninhibited neutrophil elastase in the lower respiratory tract is devastating since it causes progressive destruction of the alveolar walls. Normally, this is prevented by antiproteases which are polypeptides that inhibit proteases including elastase, usually by interacting with the catalytic site of the protease. The extracellular antiproteases that serve to protect the lung include α_1-antitrypsin (α_1-AT), secretory leukoprotease inhibitor (SLPI), α_1-antichymotrypsin, and α_2-macroglobulin (α_2-M). The most important of these are α_1-AT or α_1-PI, and SLPI (Hubbard *et al.*, 1991).

In the normal lung, the alveoli are protected against NE by α_1-AT. It is a 52-kD glycoprotein produced mainly by hepatocytes and secreted into the circulation (Vogelmeier *et al.*, 1996). However, in the course of diseases such as CF, pulmonary dysfunction occurs as a consequence of elastase inhibitor inactivation. For example, bronchoalveolar lavage fluid analysis in CF patients shows normal to high levels of α_1-AT, but even higher levels of active NE, due to the fact that α_1-AT has been oxidized and/or proteolytically cleaved by neutrophil proteases (Suter *et al.*, 1984; McElvaney *et al.*, 1991). Oxygen radical species contribute to lung destruction by increasing human leukocyte degradative activity, suggesting the existence of a synergy between elastase and oxygen radical species.

SLPI is a 12-kD protein produced by airway secretory cells which operates in concert with α_1-AT in normal individuals to protect the airways. In individuals with CF, however, the inflammatory process on the airway epithelial surface is so intense that both of these anti-NE defences are overwhelmed and rendered ineffective (Vogelmeier *et al.*, 1991) with resultant deficiency in the anti-NE protective shield of the epithelium.

α_2-Macroglobulin (α_2-M) functions as a proteinase inhibitor, as well as a carrier and regulator of the function of many cytokines (Kurdowska *et al.*, 1997). There is abundant evidence for a role of cytokines in the pathogenesis of sepsis (Beutler *et al.*, 1987; Bone, 1991). In addition to these mediators, the release of proteinases also plays an important role in the development of sepsis (Colman, 1989). Excessive activity of proteinases *in vivo* is counteracted by inhibitors, including serine proteinase inhibitors and α_2-M among others. Proteinases inhibited by α_2-M include the neutrophilic proteinase elastase and several bacterial proteinases (Maeda *et al.*, 1987). Thus, α_2-M may play an important role in regulating the hemostatic and inflammatory reactions that occur in sepsis. The inhibition of proteinases by α_2-M is thought to be due to a unique mechanism by which the proteinases cleave α_2-M at the bait region, which contains a number of peptidyl bonds that are easily hydrolyzed by various proteinases (Barret *et al.*, 1973; Harpel, 1973). Cleavage of the bait regions then induces the exposure of internal thiolester bonds, which are subsequently hydrolyzed (Sottrup-Jensen *et al.*, 1980). This latter process coincides with a change in conformation of α_2-M, which

results in entrapment of the proteinase (Barret *et al.*, 1973; Gonias *et al.*, 1983). Plasma levels of free α_2-M are reduced in patients with sepsis because α_2-M is inactivated as a result of the formation of complexes with proteinases (Duswald *et al.*, 1985; Kalter *et al.*, 1985; Seitz *et al.*, 1987; De Boer *et al.*, 1993).

In addition to a deficiency in anti-NE protection in the CF lung, there is also a deficiency in the protection against phagocyte-derived oxidants. In the healthy lung, the epithelial lining fluid contains a variety of enzymes and small molecules (e.g glutathione) capable of neutralizing oxidants (Davies *et al.*, 1991). Since neutrophils generate large amounts of oxidants, they require high intracellular concentrations of glutathione to protect themselves against injury while performing their antimicrobial or inflammatory activities. Glutathione (GSH) is a reducing agent which provides protection against the oxidation of sulphydryl groups of enzymes, globin and constituents of membranes. It also counteracts auto-oxidation of membrane lipids and helps dispose of hydrogen peroxide (Davies *et al.*, 1991). Neutrophils defective in glutathione have impaired function (Carr *et al.*, 1997).

GSH is normally present in the respiratory epithelial lining fluid (ELF) at a concentration of 150-250μM, levels 50-fold greater than those observed in plasma (Cantin *et al.*, 1987). In CF, however, the situation is different. Levels of GSH in respiratory ELF are markedly decreased (Roum *et al.*, 1990), such that the epithelium is less able to defend itself against the intense oxidant burden of CF, with resultant development of chronic, oxidant-induced damage.

The major imbalance between the large NE burden and the ineffective anti-NE defence is likely to be a key factor in the progressive destruction of lung matrix and hypersecretion in CF airways. Schuster *et al.*, (1992) have demonstrated that CF sputum is a potent secretagogue for airway submucosal glands and that this secretagogue activity is mainly due to NE activity in the sputum. NE may reach concentrations of more than 100μg/ml of sputum. CF patients may produce 150ml of sputum per day during acute exacerbations, indicating that the total amount of this enzyme is ernomous. About 90% of the endogenous inhibitor for NE, alpha$_1$-

antiproteinase inhibitor, is locally inactivated by neutrophil elastase and probably also by the oxidative attack.

3.2 *Pseudomonas aeruginosa* elastase (PE)

In addition to neutrophil elastase, *P. aeruginosa* also produces an elastase (PE) which is known to inactivate immunoglobulin G (IgG), as well as several human complement factors, and alpha-1-proteinase inhibitor (α_1-PI). PE is able to cleave human serum immunoglobulin A (IgA) and secretory IgA *in vitro*, the latter being the major immunoglobulin of the respiratory tract (Schultz and Miller, 1974; Morihara *et al.*, 1979; Doring *et al.*, 1981; Fick and Reynolds, 1983). Fibronectin plays an important role in tissue organization and its degradation by PE may account for various morphologic and functional changes in lung tissue after bacterial colonization (Azghani *et al.*, 1992). Infection of the airways by *P. aeruginosa* results in increased concentrations of proteolytic enzymes derived from both the microbial pathogen and activated phagocytes. These proteases in turn induce the degradation or inactivation (or both) of locally produced host-derived proteins, which serve in the microbial defence of the lung (Jacquot *et al.*, 1985).

PE in phosphate-buffered saline may also cause epithelial disruption without slowing CF; however, in a medium containing divalent ions, PE caused slowing of CBF as well as epithelial disruption at 100µg/ml (Amitani *et al.*, 1991). Elastase-producing strains of *P. aeruginosa* are more difficult to clear from the lungs of experimental animals than are non-elastase-producing strains (Blackwood *et al.*, 1983). Furthermore, rats with lungs already damaged by NE were unable to clear *P. aeruginosa* following experimental inoculation of the microbial pathogen into the airways (Doring *et al.*, 1988).

The afore-mentioned observations suggest that neutrophil and *Pseudomonas aeruginosa* elastases cooperate to enhance tissue destruction and promote microbial persistence.

4. PIGMENTS

4.1 Pyocyanine (Pyo)

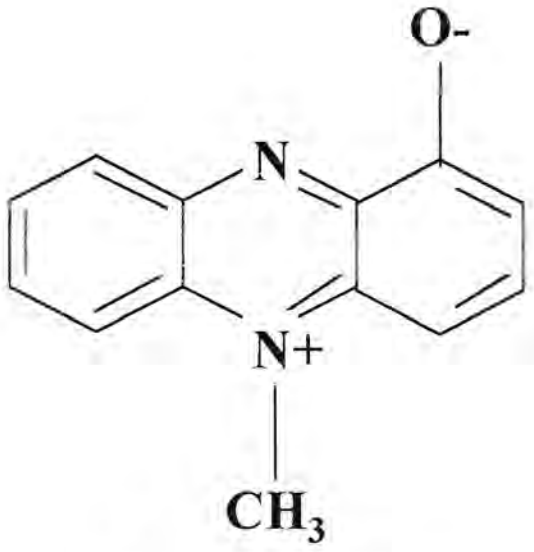
Pyocyanine and its degradation product, 1-hydroxyphenazine (1-HP), are pigments produced by *P. aeruginosa*. The molecular structures of these agents are shown in **Figure 2**. Both are virulence factors which contribute to the pathogenicity of *P. aeruginosa*. Pyocyanine (5-methyl-1-hydroxyphenazium betaine) is a blue pigment responsible for the distinctive blue colouration observed in the pus of some patients infected with *P. aeruginosa*. Its presence led to the original designation of *P.aeruginosa* as *Bacillus pyocyaneus*. The molecular weight of pyocyanine is 210.2. It forms dark blue needles in water and in the solid state it is stable for weeks in a dry and dark environment, but decomposes on long storage. Pyocyanine is zwitterionic in nature, conferring solubility in aqueous and organic solvents. It is also an electron acceptor, as well as a carrier. It is blue in its oxidized form and colourless in the reduced state. Solubility is achieved in hot water and hot ethanol.

4.2 1-Hydroxyphenazine (1-HP)

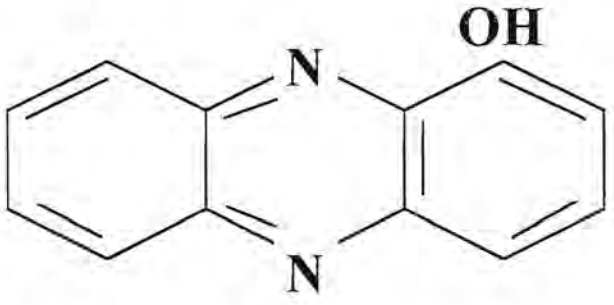
Although 1-hydroxyphenazine (1-HP) is synthesized directly by *P. aeruginosa*, it is also formed by the degradation of pyocyanine by demethylation of the 5-methyl substituent on the phenazine nucleus. 1-HP is also reffered to as hemipyocyanine or 1-phenaziol. It has a molecular weight of 196.2 and is soluble in aqueous alkaline solutions and slightly soluble in hot water.

4.3 PHENAZINE PIGMENT (Pyocyanine, 1-Hydroxyphenazine) PRODUCTION

The natural occurrence of phenazine pigments has been known since the nineteenth century (Ingram and Blackwood, 1970). The pigments contain the same basic structure as shown in **Figure 3.** Pyocyanine is produced by some strains of *Pseudomonas aeruginosa* under certain culture conditions and may be regarded as the prototype microbial phenazine pigment. It has attracted interest because of its antibiotic properties and because of its occurrence in pathogenic strains of the organism. The pigment was first isolated by Gessard (1890) following the growth of *P. aeruginosa* on complex media.



Pyocyanine



1-Hydroxyphenazine

Figure 2 : Molecular structures of pyocyanine and its degradation product, 1-hydroxyphenazine (1-HP)

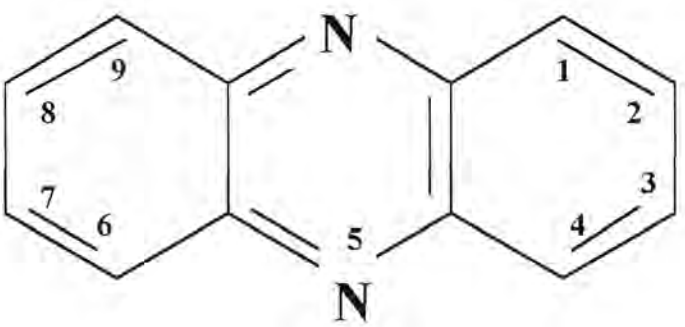


Figure 3 : Structure and numbering system of the phenazine nucleus.

The first documented evidence concerning the production of pyocyanine in a chemically defined media was that of Jordan (1899). He demonstrated the ability of some cultures to produce the pigment when grown on a mixture of salts plus asparagine or plus the ammonium salts of succinic, lactic, acetic, or citric acids. Since that time many media, usually modifications of that mentioned above, have been described in an effort to optimize the amount of pigment produced. The media usually contain glycerol as a carbon source, leucine and/or alanine as nitrogen sources, and Fe^{+2}, Mg^{+2}, SO_4^{-2}, and PO_4^{-3}, although the level of phosphate is critical and must be kept low to ensure maximum production of pyocyanine.

Studies by Valette and co-workers (1966) with the A-237 strain of *P. aeruginosa* showed that optimum pyocyanine production occurs at a concentration of 0.08M phosphate when the organism is cultured on a synthetic medium of succinic acid and ammonium chloride. Halpern *et al.*, (1962) studied the production of pyocyanine

in resting cell suspensions. Growth as measured by optical density at 490nm occurred while the production of pyocyanine proceeded. When the effects of inhibitors of the production of pyocyanine were studied, it was found that inhibitors which produce inhibition of growth also inhibited the production of the pigment. The production of pyocyanine commences when the phosphate concentration reaches a critical minimum level and terminates as the phosphate level rises.

Millican (1962) using a direct label incorporation, or an isotope dilution procedure, showed that quinic or shikimic acids serve as good sources of pyocyanine carbon. However, in each study, interpretation was complicated by bacterial growth indicating that the carbon source was metabolized prior to incorporation into pyocyanine. The problem of precursor metabolism prior to incorporation into pigment was circumvented by using a mutant, Q^-, which was unable to oxidize and hence, unable to metabolize shikimic and quinic acids (Ingledew and Campbell, 1969). When cultures of the wild type, W^+, were added to a resuspension medium containing 2-ketogluconate-^{14}C as the initial sole source of carbon, it was found by isotope dilution that 9% of the pyocyanine

carbon was derived from shikimic acid. However, when the same experiment was repeated, with the Q^- strain, 98% of the pyocyanine ring carbon was derived from shikimic acid, whereas 40% was derived from quinic acid. The authors also reported that quinic acid is not a direct precursor of pyocyanine carbon. Their data strongly suggested that quinic acid is converted to shikimic acid, which is then incorporated into the pyocyanine molecule.

Other authors have, however, reported that glycerol appears to be the primary carbon source utilized for pigment production. These observations concerning the biosynthetic pathway for phenazine pigment production indicate that glycerol and shikimic acid are for the most part involved.

Certain cultures of *P. aeruginosa* also produce 1-hydroxyphenazine, the product of pyocyanine demethylation (Schoental, 1941). Other monohydroxyphenazines namely 2-hydroxyphenazine and 2-hydroxyphenazine-1-carboxylic acid have also been obtained as minor products following culture extraction and isolation procedures.

4.4 EFFECTS OF PYOCYANINE AND 1-HYDROXYPHENAZINE ON NEUTROPHIL FUNCTION

Pyocyanine at 50µM has been reported to inhibit phorbol myristate acetate (PMA) induced superoxide generation by neutrophils by 60%, possibly by interfering with the ubiquinone-cytochrome b site of the electron transport chain (Armstrong *et al.*, 1971). Lower pyocyanine concentrations (1µM) on the other hand increased superoxide production by neutrophils. Neutrophil viability, granule content and degranulation, complement receptor translocation and expression appeared not to be altered (Berger *et al.*, 1986).

Other authors have also reported that pyocyanine significantly affects the generation of superoxide by human neutrophils in a dose-dependent manner (Miller *et al.*, 1987). Pyo at 50µM inhibited PMA-induced superoxide generation by approximately 30%, while lower concentrations of less than 5µM produced a 200% enhancement of superoxide generation. In these experiments, pyocyanine did not activate superoxide

generation in the absence of PMA (Miller *et al.*, 1987).

Sorensen *et al.*, (1987) reported that 1-hydroxyphenazine at high concentrations (50µM) causes moderate inhibition of superoxide production. However, the effect of *P. aeruginosa* culture supernatants on PMA-induced superoxide generation was quite variable, ranging from 50% inhibition to 50% enhancement. If similar variations occur *in vivo*, high concentrations may inhibit superoxide generation and bacterial killing, while lower concentrations may act synergistically with other stimuli to increase superoxide generation and oxygen radical mediated tissue damage (Sorensen *et al.*, 1987).

More recently, Ras *et al.*, (1990) reported that pyocyanine, but not 1-HP, increased the generation of superoxide, as well as the rate and duration of oxygen consumption by activated neutrophils. However, 1-HP had a greater effect than pyocyanine on the release of myeloperoxidase and lysozyme from activated neutrophils. Ras *et al.*, (1992) also reported that 1-HP, but not pyocyanine, caused a dose-related enhancement of the release of elastase from N-formyl-L-methionyl-L-leucyl-L-phenylalanine in combination with cytochalasin-B (FMLP:CB)-stimulated neutrophils confirming, their previous finding in which 1-HP increases the mobilization of primary granules. The biochemical mechanism by which 1-HP mediates these pro-inflammatory interactions with neutrophils has yet to be established.

4.5 EFFECTS OF PYOCYANINE, 1-HYDROXYPHENAZINE AND *Pseudomonas* ELASTASE ON CILIATED RESPIRATORY EPITHELIUM.

Ciliated epithelial cells play a major role in preserving the functional integrity of the airways (Devalia *et al.*, 1992) with mucociliary clearance being the first-line defence mechanism of the human respiratory tract against inhaled particles, including bacteria. Defective mucociliary clearance is associated with recurrent chest infections (Wilson *et al.*, 1987). *Pseudomonas aeruginosa* culture filtrates obtained after 18hrs of culture have been shown to slow human ciliary beat frequency of nasal ciliated epithelium *in vitro*. Pyocyanine accounted for a significant proportion of this activity. Concentrations of 20µM or more of pyocyanine caused a gradually increasing ciliostasis over a period

of 4hours. In contrast, 10-50μM of 1-HP had a dose-dependent, immediate inhibitory effect on ciliary beat frequency, which gradually diminished over a 4hr observation period (Wilson *et al.*, 1985; Wilson *et al.*, 1987). It has also been found that the slowing of ciliary beat frequency is associated with a significant fall in intracellular adenosine 3', 5'-monophosphate (cAMP) (90%) and ATP (66%), and that the effect was reversible after pyocyanine was removed by washing, or by exposure of the ciliated respiratory epithelium to cAMP-elevating agents (Kanthakumar *et al.*, 1993).

In a study carried out by Smallman *et al.*, (1984) cilia incubated in PE positive secretions, showed a considerable decrease in ciliary beat frequency over six hours, falling from a mean of 13.40 beats/second to 6.78 beats/second ($p<0.001$). This clearly shows that, elastase is capable of inhibiting ciliary activity, probably by damaging the respiratory epithelium.

These observations demonstrated that bacterial products, specifically pyocyanine, 1-HP and elastase, released from *P. aeruginosa*, impair ciliary activity, causing a fall in ciliary beat frequency, disruption of epithelial integrity and the slowing of tracheal mucociliary transport (Wilson *et al.*, 1987; Munro *et al.*, 1989).

4.6 EFFECTS OF PYOCYANINE AND 1-HYDROXYPHENAZINE ON LYMPHOCYTE FUNCTION

Killed *P. aeruginosa*, cell-free *P. aeruginosa* culture supernatants, and the purified *P. aeruginosa* phenazine pigment pyocyanine, have all been shown to inhibit mitogen-induced human lymphocyte proliferation as measured by [^{3}H]-methyl-thymidine ([^{3}H]TdR) uptake *in vitro* (Sorensen *et al.*, 1983). In the early stages of T-cell activation mediated by the calcium ionophores A23187 and ionomycin, pyocyanine was found to decrease the production of the critical cytokine IL-2 and also to inhibit the expression of the IL-2 receptor (Nutman *et al.*, 1987). However, Muhlradt *et al.*, (1986) did not detect inhibition of IL-2 production by pyocyanine-treated murine T-cells, which is contradictory to the report by Nutman *et al.*, (1987). In 1990, however, Ulmer *et al.*, reported that pyocyanine has both stimulatory and inhibitory effects on the proliferative responses of activated lymphocytes. At concentrations of 0.1μg/ml or less, the

proliferation of T- and B-lymphocytes was enhanced, but at 0.5µg/ml or higher, the proliferation was inhibited, while IL-2 production by T-lymphocytes was enhanced at concentrations of up to 0.5µg/ml, but totally inhibited at 1.0µg/ml. It can be concluded from these findings that pyocyanine has immunomodulatory properties, either immunosuppressive or immunostimulatory, which are related to the concentration of the pigment.

4.7 EFFECTS OF PYOCYANINE ON OTHER CELL TYPES

Pyocyanine increases oxidant stress, and because oxidant stress has been shown to affect cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$) in other cell types (Masumoto *et al.*, 1990; Roveri *et al.*, 1992; Dreher and Junod, 1995), Denning *et al.*, (1998) investigated the effects of pyocyanine on $[Ca^{2+}]_c$ in human airway epithelial cells. They reported inhibition of 1,4,5-triphosphate (IP_3) formation and $[Ca^{2+}]_c$ increases in response to G protein-coupled receptor agonists by pyocyanine at low concentrations. Conversely, at higher concentrations, pyocyanine increases $[Ca^{2+}]_c$. The pyocyanine-dependent $[Ca^{2+}]_c$ increase appears to be oxidant dependent and to result from increased inositol triphosphate and release of Ca^{2+} from intracellular stores. By disrupting Ca^{2+} homeostasis, pyocyanine could interfere with the critical functions of airway epithelium (ion transport, mucus secretion, and ciliary beat frequency) and thus contribute to the pathophysiological effects observed in *Pseudomonas*-associated lung disease.

5. CALCIUM AND cAMP AS INTRACELLULAR MESSENGERS

All cells rely on the production and detection of signalling molecules to function and survive. Cell proliferation and differentiation need to be regulated and stringently coordinated, as do the cell's metabolic processes. Calcium mobilization, the transient elevation of cAMP, protein phosphorylation, and lipid turnover, are all involved in transmembrane signalling and modulation of the functions of various cell types. Calcium (Ca^{2+}) and cAMP are the two most widespread messenger systems utilized in the signal transduction pathways of eukaryotic cells. The two can also interact with each other.

Adenosine 3', 5'-monophosphate (cAMP) was identified by Sutherland and Rall in 1958

as an intracellular second messenger of hepatic glycogenolysis and subsequently for a variety of hormones, inflammatory mediators and cytokines, and has been shown to modulate models of immune and non-immune inflammation *in vivo* and a variety of cellular processes *in vitro*. cAMP is generated from adenosine triphosphate (ATP) by the receptor-mediated activation of adenylate cyclase. Rises in cAMP are usually transient (Moore and Willoughby, 1995). cAMP is metabolized to a physiologically inactive form (5'-AMP) by phosphodiesterase (PDE) enzymes which are inhibited by methylxanthines such as theophylline and caffeine. Both PDE inhibitors augment hormonal and transmitter effects mediated by cAMP. Many neurotransmitters and hormones also act by opening ligand-dependent Ca^{2+} channels, either directly or indirectly via cAMP or by releasing Ca^{2+} from intracellular stores such as the endoplasmic reticulum via inositol triphosphate receptors.

There is normally 10^{-7}mol/L of Ca^{2+} in the cell and 5mmol/L extracellularly. This amazing differential is maintained by the cell membrane and by the ATP-driven Ca^{2+} pump which regulates Ca^{2+} influx. Most of the Ca^{2+} in the cell is compartmentalized or is bound to cellular constituents such as membranes and binding proteins. Ca^{2+} may also be stored in the nucleus on binding proteins, or be transported across the endoplasmic reticulum. Ca^{2+} regulates many cell functions by influencing the activity of Ca^{2+}-binding proteins such as troponin and calmodulin (Guyton, 1991). These proteins in turn control pathways that determine the activity of the cell, for example, contraction and secretion. Not only does Ca^{2+} via calmodulin regulate enzymes involved in the synthesis and degradation of cAMP, but the two intracellular messengers nearly always function together. When the stimulant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) binds to specific neutrophil membrane receptors, the result is receptor-G-protein coupling, followed by the activation of phospholipase C (PLC) and generation of inositol triphosphate (IP_3) which is a product of the hydrolysis of phosphatidylinositol-4,5-biphosphate (PIP_2) (Di Virgilio *et al.*, 1985; Lew *et al.*, 1986). IP_3 then binds to receptors on the endoplasmic reticulum, or in the case of neutrophils, to specialized Ca^{2+}-storage organelles known as calciosomes, with resultant elevation in the cytosolic concentration of the cation (Prentki *et al.*, 1984; Di Virgilio *et al.*, 1985; Krause *et al.*, 1989; Yan *et al.*, 1995). These events which are summarized in **Figure**

4, are extremely rapid, occurring within less than one second after the ligand-receptor interaction (Tashjian *et al.*, 1987; Wyman *et al.*, 1987).

In neutrophils, a variety of agonists including chemotactic peptides (Baggioloini *et al.*, 1993), interleukin 8 (Elsner *et al.*, 1992), C5a (Gerard *et al.*, 1994), cross-linking of integrins (Petersen *et al.*, 1993) and other mechanisms, trigger an increase in cytosolic free calcium $[Ca^{2+}]_c$. The cytosolic free calcium concentration is crucial for the control and the regulation of many cellular functions. The increase in $[Ca^{2+}]_c$ is apparently the result of both the entry of extracellular Ca^{2+} via membrane channels and release of Ca^{2+} from intracellular stores (Favre *et al.*, 1996). The calcium influx that is activated through the depletion of intracellular Ca^{2+} stores and not through plasma membrane depolarization is termed "store-operated Ca^{2+} influx". Store-operated Ca^{2+} influx may be detected with a variety of techniques including the use of Ca^{2+}-sensitive intracellular dyes like Fura-2/AM, or alternatively by radiometric procedures using radiolabelled Ca^{2+} (Favre *et al.*, 1996).

Radiometric detection of cell-associated ^{45}Ca in combination with Fura-2 fluorescence procedures is extremely useful when measuring Ca^{2+} fluxes in FMLP-activated human neutrophils (Anderson *et al.*, 1997). Pre-incubation of neutrophils in medium containing ^{45}Ca as the sole source of Ca^{2+}, followed by activation with FMLP, resulted in a rapid efflux of the cation, which coincided with its release from intracellular stores. Efflux terminated at around 30s after addition of FMLP to neutrophils and resulted in the loss of 42±3% of cell-associated ^{45}Ca. Net influx of ^{45} Ca, which was insensitive to the voltage-dependent Ca^{2+} channel blockading agent, verapamil (20μM), could only be detected at 30-60s after the addition of FMLP to neutrophils, and proceeded for about 5min, resulting in intracellular concentrations of Ca^{2+} which were 27±3% higher than preactivation levels (Anderson *et al.*, 1997). These results demonstrated that the efflux of cytoplasmic Ca^{2+} mobilized from intracellular stores during activation of neutrophils by FMLP, and the subsequent influx of extracellular Ca^{2+} to replete these stores, are chronologically distinct events in FMLP-activated neutrophils (Anderson *et al.*, 1997).

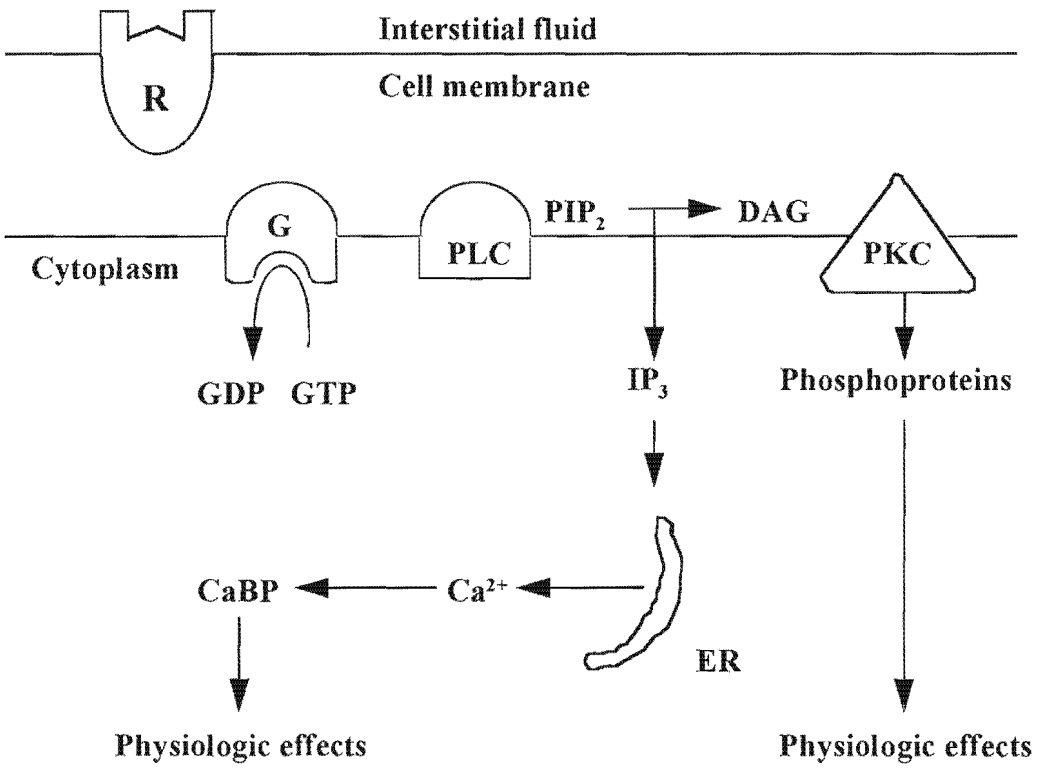


Figure 4: Diagrammatic representation of release of inositol triphosphate (IP_3) and diacylglycerol (DAG) as second messengers. Binding of ligand to receptor (R) activates phospholipase C (PLC) via a G protein. The resulting hydrolysis of PIP_2 produces IP_3, which releases Ca^{2+} from the endoplasmic reticulum (ER), and DAG, which activates protein kinase C (PKC), CaBP and Ca^{2+}-binding proteins.

6. cAMP-BASED ANTI-INFLAMMATORY STRATEGIES

It has been realized for more than two decades that physiologic (E-series prostaglandins, adenosine, catecholamines) and pharmacologic (β_2-adrenoreceptor agonists, phosphodiesterase inhibitors, adenosine receptor agonists) cAMP-elevating agents possess broad-spectrum anti-inflammatory properties affecting the production and/or release of many inflammatory mediators (leukotrienes, PAF, oxidants, granule enzymes, chemokines and cytokines, especially TNF-α) encompassing a range of different immune and inflammatory cells (neutrophils, eosinophils, macrophages, mast cells, B-lymphocytes and T-lymphocytes). These anti-inflammatory and immunomodulatory properties of cAMP-elevating agents have recently been reviewed by Moore and Willoughby (1995).

Despite the well-accepted anti-inflammatory potential of cAMP-elevating agents, their clinical application has been thwarted by the lack of availability of agents which selectively target immune and inflammatory cells. This situation may change, however, with the acquisition of selective adenosine type A2 receptor agonists and second generation type 4 phosphodiesterase inhibitors, which may selectively target inflammatory cells (Torphy and Undem, 1991; Underwood *et al.*, 1998).

7. cAMP-ELEVATING AGENTS

cAMP-elevating agents are those agents that increase the levels of intracellular cAMP via different pathways. Several diverse groups of pharmacological agents such as β_2-adrenoreceptor agonists, phosphodiesterase (PDE) inhibitors and agonists of adenosine type 2a (A2a) receptors have this property, as does dibutyryl cAMP, a cell-permeable analogue of cAMP.

7.1 Dibutyryl cAMP

Dibutyryl cAMP, although not a pharmacological agent, is an analogue of cAMP. It is less polar and therefore more cell-permeable than cAMP.

7.1.1 Anti-inflammatory activities of dibutyryl cAMP

In addition to regulating neutrophil function, dbcAMP can also regulate ciliary activity

in human respiratory epithelium. Nasal respiratory epithelial strips were brushed from the inferior nasal turbinates of awake adults, anaesthesized children and anaesthesized adults and ciliary beat frequency measured in the absence and presence of dbcAMP *in vitro*. It was found that incubation of human nasal epithelium in the presence of dbcAMP, significantly increased ciliary beat frequency of epithelial cells (Di Benedetto *et al.*, 1991).

DbcAMP has also been used in combination with cAMP-elevating agents and the following anti-inflammatory effects have been demonstrated *in vitro* i) inhibition of O_2^- production by human neutrophils through the action of cAMP-dependent protein kinase ii) induction of human thioredoxin (oxidative stress-inducible protein with protective function) when retinal pigment epithelial cells were pretreated with 10μM prostaglandin E1 (Yamamoto *et al.*, 1997), and iii) reduction of actin polymerization (involved in the mechanical functioning of the neutrophils) by adenosine in neutrophils via a cAMP-dependent pathway (Zalavary *et al.*, 1998).

7.2 Beta$_2$-adrenoreceptor agonists

Beta$_2$-adrenoreceptor ($ß_2$-adrenoreceptor) agonists are the most effective broncho-dilators currently available and exhibit efficacy irrespective of the mediator(s) evoking bronchospasms (Giembicz, 1996). They are widely used in the treatment of asthma and chronic obstructive pulmonary disease. The interaction of a β_2-adrenoreceptor agonist with its cognate receptor, leads to the liberation of the α-subunit of the stimulatory G-protein (G_s), which in turn stimulates adenylate cyclase, resulting in an increase in cAMP.

7.2.1 Salmeterol and salbutamol

Salmeterol and salbutamol are the prototype long- and short-acting β_2-adrenoreceptor agonists respectively used in the treatment of asthma. Their bronchodilatory action helps in the relaxation of airway smooth muscle. Bronchodilators are presumed to act by reversing contraction of airway smooth muscle and the molecular basis of bronchodilation involves an increase in intracellular cAMP and a reduction in cytosolic calcium ion concentration (Barnes, 1996). Structurally, salmeterol is related to

salbutamol, the major difference being that salmeterol also possesses an extended lipophilic side chain (Johnson *et al.*, 1993). The lipophilic nature of the salmeterol molecule allows it to be concentrated into the membrane lipid, from where the molecules slowly access the β_2-adrenoreceptor. The suggestion has been made that, due to the lipophiliicity of salmeterol, non-specific physicochemical interactions with the plasma membrane rather than β_2-agonist effects may be responsible for some of the anti-inflammatory effects of this agent (Anderson *et al.*, 1996; Nials *et al.*, 1997).

With respect to cAMP-mediated anti-inflammatory effects, these are achieved via interactions of β_2-agonists with β_2-adrenoreceptors on vascular endothelium, respiratory epithelium or on mast cells (Fugner, 1989; Erjefalt and Persson, 1991; Advenier *et al.*, 1992; Whelan *et al.*, 1993; Kanthakumar *et al.*, 1993).

7.2.1.1 Anti-inflammatory effects of salbutamol

Fantozzi *et al.*, (1984) reported inhibition of the respiratory burst and lysosomal enzyme release in FMLP-stimulated neutrophils by salbutamol and other β_2-agonists, which was prevented by pretreatment of the cells with β-antagonists. This is in agreement with the inhibitory activities of salmeterol on alveolar macrophages (Baker and Fuller, 1990; Johnson *et al.*, 1993) and T-lymphocytes (Nathan, 1987; Sekut *et al.*, 1995).

Inhaled salbutamol (300μM) blocks pulmonary neutrophil sequestration and prevents lung function abnormalities (Masclans *et al.*, 1996). It also increases the ciliary beat frequency (CBF) of bronchial epithelial cells significantly, which is associated with a significant increase in cAMP (Masclans *et al.*, 1996).

7.2.1.2 Anti-inflammatory effects of salmeterol

Salmeterol inhibits the respiratory burst of activated human neutrophils in a dose-dependent manner. This was observed when salmeterol was added to neutrophils immediately before exposure to FMLP and resulted in a dose-dependent inhibition of the total cumulative amount of superoxide produced by the cells during a 3hr period of incubation (Ottonello *et al.*, 1996).

In yet another study (Anderson *et al.*, 1996), concentrations (>1μM) of salmeterol higher than those required to activate β_2-receptors were found to neutralize the pro-oxidative interactions of the bioactive phospholipids (LPC, PAF and LPAF) with neutrophils without affecting their synthesis by these cells. It was concluded that salmeterol antagonizes the pro-inflammatory, pro-oxidative activity of several bioactive lipids implicated in the pathogenesis of bronchial asthma, by a mechanism related to the membrane-stabilizing, rather than to the β_2-agonist properties of this agent.

Numerous studies have shown that bronchial eosinophil infiltration and activation play a critical role in the pathogenesis of bronchial asthma (Wardlaw *et al.*, 1988; Barnes, 1989; Ezeamuzie *et al.*, 1997). Exposure of eosinophils to platelet-activating factor (PAF), interleukin-5 (IL-5) or PMA results in concentration-dependent generation of superoxide. Superoxide production by eosinophils in response to PAF and IL-5 was strongly inhibited by salmeterol in contrast to that induced by PMA. This stimulus-dependent inhibition may be due to the different signal transductional pathways utilized by the different stimuli. PAF and IL-5 are perhaps the most important mediators of allergic inflammation in asthmatic lungs. The ability of salmeterol to strongly inhibit eosinophil adherence and O_2^- release by these two stimuli suggests that these effects might significantly contribute to the anti-inflammatory properties of this drug *in vivo*. On the other hand, PMA is a direct PKC activator that is slowly metabolized by the cells and therefore causes sustained activation of PKC which is more difficult to block, even with the long-acting β_2-adrenoreceptor agonist, salmeterol.

Assem and Schild (1969) and Undem *et al.*, (1988) have also shown that β-adrenoreceptor agonists are effective inhibitors of the stimulated release of histamine from human lung mast cells (HLMC). Human lung mast cells were incubated in either buffer or 0.1μM of salmeterol or salbutamol for 15min before challenging them with various concentrations of anti-IgE. Both salmeterol and salbutamol caused a statistically significant reduction in histamine release from HLMC. The mechanism by which salmeterol acts to inhibit HLMC responses is said to be complex, perhaps involving both the β-adrenoreceptor-dependent and independent mechanisms (Chong *et al.*, 1998).

One characteristic feature of the action of salmeterol, both *in vivo* and *in vitro*, is its long duration of action (Nials *et al.*, 1993; Tattersfield, 1993). Studies designed to investigate the effects of salmeterol on smooth muscle relaxation (Nials *et al.*, 1993) and inhibition of mediator release from lung fragments (Butcher *et al.*, 1991), showed that the relaxant and inhibitory effects of salmeterol persisted even after the tissues had been washed.

At high concentrations, salmeterol also has effects which are not mediated by β_2-adrenoreceptors, e.g. inhibition of thromboxane B_2 release from alveolar macrophages (Baker & Fuller, 1990), relaxation of guinea-pig gastric fundus (Baker *et al.*, 1994) and inhibition of electrically-induced contractions in ferret trachea (Bergendal *et al.*, 1992). However, the mechanism(s) underlying these non-β_2-adrenoreceptor mediated responses is still not defined. Membrane-stabilizing activity may play a role.

Because of their relatively low affinity and low expression of β_2-adrenoreceptors on inflammatory cells (Barnes, 1995), salbutamol and salmeterol have limited application as systemic anti-inflammatory agents and are primarily used as locally-administered bronchodilators.

7.3 Phosphodiesterase inhibitors

Phosphodiesterases (PDEs) are a family of enzymes which metabolize, and thereby inactivate the naturally occurring second messenger nucleotide 3',5-cyclic monophosphate (cAMP).There are 8 distinct PDE-inhibitor isoenzyme profiles known to date (Giembycz and Dent, 1992; Barnes, 1996; Torphy, 1998). The classification is based on their protein and gene-encoding DNA sequences. The enzymes differ with respect to substrate selectivity, sensitivity to calcium/calmodulin, allosteric regulation by cGMP, sensitivity to phosphorylation and distribution both in tissue and subcellular compartments (Livi *et al.*, 1990). Each family contains subfamilies, and further diversification may arise through genes that can give rise to two or more alternatively spliced RNAs. Tissues may express more than one family of PDEs, but in inflammatory cells (neutrophils, eosinophils and monocytes), members of the PDE type 4 family are dominant (Moore and Willoughby, 1995; Torphy, 1998)).

PDE 4 enzymes are cAMP-specific, calcium/calmodulin-independent, and are not regulated by cGMP (Torphy, 1998). Compounds which possess PDE type 4 inhibitory activity, are able to elevate intracellular cAMP levels and to down-regulate oxidant generation by activated neutrophils (Bevilacqua *et al.*, 1994). In addition to inflammatory cells, PDE 4 enzymes are also found in the liver, brain, smooth muscles, heart and kidney (Moore and Willoughby, 1995). PDE inhibitors possess the potential to interfere with the functions of nearly every cell type present in the airway tissue (Schudt *et al.*, 1995).

Inhibitors of PDE fall into two distinct groups viz; those which are non-selective, typified by theophylline and its derivatives and those which selectively affect one isoenzyme type e.g. rolipram, a selective inhibitor of type 4 PDE.

7.3.1 Theophylline

Theophylline has long been used in the treatment of asthma. Theophylline mediates relaxation of smooth muscle and also possesses anti-inflammatory and immunomodulatory properties. The molecular mechanism of bronchodilation is almost certainly related to PDE-inhibition, resulting in an increase in cAMP (Pauwels, 1987; Persson, 1988; Banner and Page, 1996).

7.3.1.1 Anti-inflammatory effects of theophylline

The anti-inflammatory interactions of theophylline, as well as those of isoenzyme selective PDE-inhibitors with different cell types involved in inflammatory responses are well-recognized. These include mast cells, basophils, lymphocytes, eosinophils, neutrophils, monocytes and natural killer cells (Banner and Page, 1996).

Human lung mast cells release a variety of bronchoconstrictor mediators such as histamine, leukotriene C_4 and prostaglandin D_2, and these mediators have been suggested to be the predominant mediators of allergen-induced bronchoconstriction in allergic asthma. Theophylline has been shown to reduce both antigen and anti-immunoglobulin E (IgE) receptor antibody-stimulated histamine release from rat peritoneal mast cells (Pearce *et al.*, 1982), and to reduce histamine release from

human basophils (Louis and Radermecker, 1990). However, these effects are only seen at very high concentrations of theophylline.

T-lymphocytes directly recognize and respond to processed antigens and are involved in many stages of the allergic response, including the regulation of IgE production by B-lymphocytes, as well as the recruitment of other inflammatory cells such as eosinophils into the airways. Incubation of peripheral blood lymphocytes with theophylline suppresses these pro-inflammatory activities of T-lymphocytes (Zocchi *et al.*, 1985). It has also been reported that therapeutic levels of theophylline (10-20µg/ml) inhibit the tumoricidal activity of natural killer cells (Coskey *et al.*, 1993).

Coincubation of human neutrophils with theophylline at 10-50µM has been reported to result in inhibition of the respiratory burst and generation of arachidonic acid metabolites induced by chemotatic peptides, such as N-formyl-met-leu-phe (FMLP) and the calcium ionophore A23187 (Nielson *et al.*, 1986). Type 4 PDE inhibitors, at concentrations which inhibited the degradation of cAMP, have also been found to inhibit the neutrophil respiratory burst (Nielson *et al.*, 1990; Dent *et al.*, 1991).

Clinically, treatment of asthmatic children for 10 days with theophylline is associated with inhibition of chemotaxis of neutrophils and mononuclear cells *ex vivo* (Contino-Neto *et al.*, 1991).

7.3.2 Rolipram

Rolipram [4-(3-cylopentyloxy-4-methoxyphenyl)-2-pyrrolidine] or ZK 62711 (Schwabe *et al.*, 1976) is the selective, potent prototype inhibitor of PDE type 4. It has been shown to increase cAMP in a variety of cell types including inflammatory cells (Donaldson *et al.*, 1988; Marivet *et al.*, 1989).

7.3.2.1 Anti-inflammatory effects of rolipram

Bacterial lipopolysaccharide (LPS) has many proinflammatory actions in the lung. These include the induction of neutrophil sequestration in pulmonary capillaries, upregulation of cell adhesion molecules on endothelial cells, and the promotion of

cytokine synthesis and release from alveolar macrophages and endothelial cells (Libby *et al.*, 1986; Pober *et al.*, 1986; Schleimer *et al.*,, 1986; Nathan, 1987). One of the major cytokines induced by LPS is tumor necrosis factor alpha (TNFα). TNFα has been implicated as a mediator of the pathologic changes encountered in septic shock. This is because TNFα levels are elevated in the plasma, bronchoalveolar lavage (BAL) fluid, and lung tissue of patients with acute respiratory distress syndrome (Marks *et al.*, 1990; Roten *et al.*, 1991).

Agents which increase cAMP, such as PDE-inhibitors, inhibit TNFα production both *in vitro* and *in vivo* (Renz *et al.*, 1988; Kunkel *et al.*, 1989; Endres *et al.*, 1991). Inhibition of the PDE enzymes results in an accumulation of intracellular cAMP which leads to suppression of the proinflammatory activity of neutrophils, including chemotaxis, degranulation and the respiratory burst (Lad *et al.*, 1985; Nielson *et al.*, 1990).

In a study reported by Miotla *et al.*, (1998), acute lung injury was induced in mice by combined intravenous administration of LPS (10mg/kg) and zymosan (3mg/kg). Pretreatment of the mice with rolipram (5mg/kg, intraperitoneally), was found to protect against the induction of lung injury by the combination of LPS and zymosan, while extravascular albumin accumulation in the lungs was reduced by 89%. Neutrophil sequestration, as assessed by MPO activity was also significantly reduced, as were levels of TNFα. These findings are in agreement with the report by Turner *et al.*, (1993) that rolipram attenuates LPS-induced mortality and gross pulmonary injury in rats as a result of the suppression of the increase in serum TNFα levels. With respect to *in vitro* studies, rolipram has been reported to inhibit the activation of phospholipase A_2 (PLA_2) and phospholipase D (PLD) (Nakashima *et al.*, 1995), as well as the production of leukotriene B_4 by human monocytes (Griswold *et al.*, 1993), while the inhibitory effects of this agent on the prooxidative activities of human neutrophils and the release of granule enzymes by these cells are well-recognized (Santing *et al.*, 1995; Sullivan *et al.*, 1995; Berends *et al.*, 1997; Miotla *et al.*, 1998).

7.3.4 Adenosine receptor agonists

Both adenosine and adenine nucleotides are found in all living cells as part of the

normal metabolic machinery. Their concentrations are dynamically regulated by a variety of pathophysiological conditions (Berne, 1963; Olsson *et al.*, 1990). Under conditions of stress, such as hypoxia, when the cellular energy state is depressed, intracellular adenosine levels acutely increase and adenosine is released from the cell. Adenosine, once released, can activate adenosine receptors, which in turn regulate a diverse set of physiological functions (Olsson *et al.*, 1990; Stiles, 1992). Adenosine is known to promote intracellular cAMP accumulation in a variety of cells and this is a critical physiological function of this agent.

There are different adenosine receptor subtypes, which are differentiated on the basis of the effect of adenosine on cellular content of cAMP. These include the A1, A2a, A2b and A3 receptor subtypes, each of which display specific properties (Fredholm *et al.*, 1994). Molecular cloning of A1 and A2 receptors has revealed that they both belong to the superfamily of guanine nucleotide-binding regulatory protein (G-protein)-coupled receptors (Zhou *et al.*, 1992). The A1 receptors which are coupled to the G_i molecule of the G-protein inhibit adenylyl cyclase (Van Calker *et al.*, 1979; Freissmuth *et al.*, 1991; Munshi *et al.*, 1991). A2 receptors on the other hand, are coupled to the G_s proteins and activate adenylyl cyclase (Stiles, 1986; Cronstein *et al.*, 1988).

7.3.4.1 Adenosine A1 receptors

Adenosine A1 receptors are widely distributed throughout the nervous system, with the highest concentration in the cortex, hippocampus and the cerebellum (Goodman and Snyder, 1982; Reddington and Lee, 1991). They are present in both pre- and postsynaptic regions of the central nervous system. On presynaptic terminals, their main action is to limit the availability of calcium to the excitation-secretion coupling mechanism involved in the exocytotic release of neurotransmitters (Harms *et al.*, 1979; Ribeiro, 1995). Postsynaptically, adenosine A1 receptors usually induce hyperpolarization of cells, at least partly by opening potassium channels (Trussel and Jackson, 1985; Thompson *et al.*, 1992). An example of an A1 adenosine receptor agonist, CPA is shown in **Figure 5.**

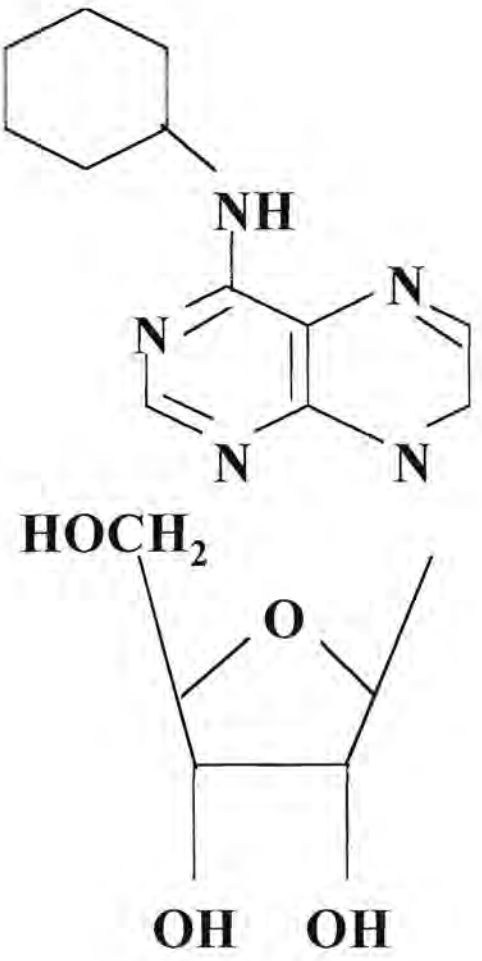


Figure 5 : Molecular structure of cyclopentyladenosine (CPA)

7.3.4.2 Adenosine A2 receptors

A2 receptors are found throughout the CNS, with higher concentrations of the A2a subtype in the striatum, and nucleus accumbens (Jarvis *et al.*, 1989; Jarvis and Williams, 1989) as well as on immune and inflammatory cells (Van Calker *et al.*, 1979; Church *et al.*, 1983; Hughes *et al.*, 1984; Hoffman *et al.*, 1997; Huang *et al.*, 1997). They are glycoproteins with molecular mass around 45kD (Barrington *et al.*, 1990). The A2 receptor has been subclassified on the basis of the differences in binding affinities for adenosine analogues (CGS21680 and NECA). CGS21680, which has previously been shown to have high affinity for the rat striatal A2a receptor (Bruns *et al.*, 1986; Jarvis *et al.*, 1989), also exhibited a high degree of binding to the A2 receptors of human neutrophils, lymphocytes and platelets. However, in guinea pig aorta, CGS21680 was markedly less active than NECA (A2 receptors). It was therefore proposed that CGS21680 could be used with NECA as a reference to differentiate A2a from either A2b or A1 receptors (Hutchinson *et al.*, 1990; Gurden *et al.*, 1993). The molecular structure of CGS21680 is shown in **Figure 6.** A highly conserved homology in the amino acid sequence has been reported between A2a and A2b subtypes. The receptor entities are quite similar in their transmembrane parts, but differ in the carboxy terminal domain, with a larger tail for the A2a receptor, the functional significance of which remains unclear (Fredholm *et al.*, 1994).

Accumulation of extracellular and intracellular adenosine in adenosine deaminase activity is lymphotoxic and results in severe combined immunodeficiency (SCID). The severity of the disease is dependent on the level of residual adenosine deaminase activity and, by implication, on the concentration of accumulated adenosine. Humans with inherited adenosine deaminase activity deficiency are severely immunocompromised and infantile death results from recurrent infections due to the accompanying lymphopenia and the absence of T- and B-cell immunity (Kellems *et al.*, 1985; Hirschhorn *et al.*, 1995; Huang *et al.*, 1997). ADA deficiency results in dramatically decreased number of peripheral lymphocytes in lymph nodes and spleen. Immune dysfunction in the ADA variant of SCID is thought to result from the chronic elevation in circulating levels of adenosine which in turn causes sustained activation of A2a receptors on immune and inflammatory cells leading to high intracellular concentrations of cAMP and decreased activity of these cells.

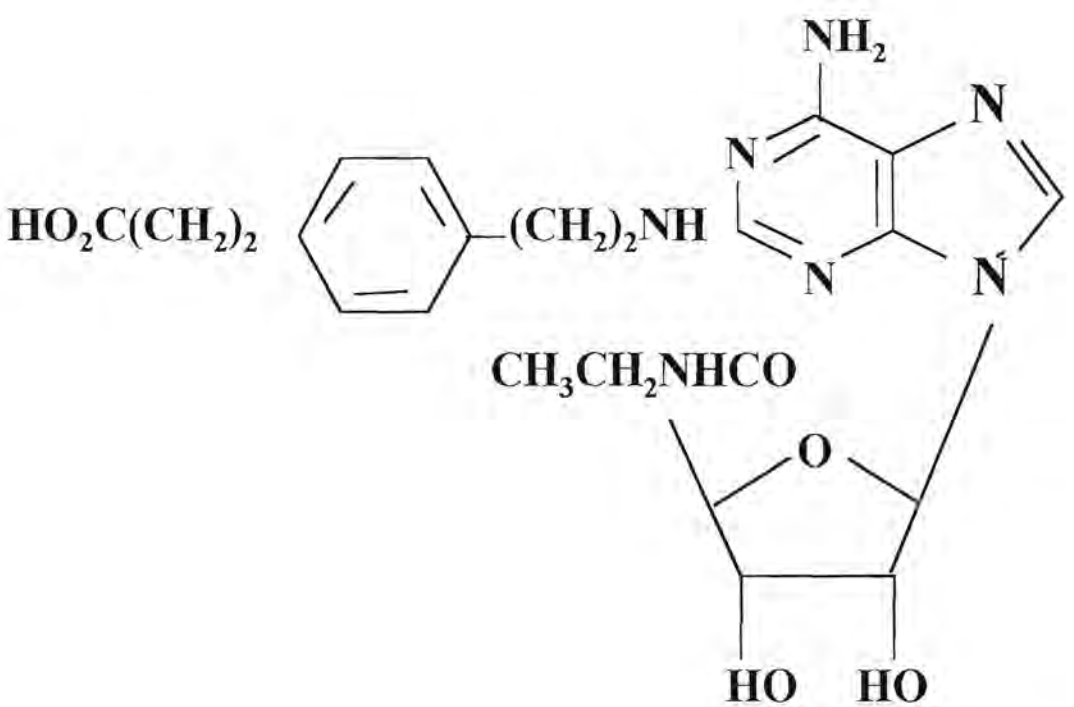


Figure 6: Molecular structure of 2-[p-(2-carboxyethyl)phenylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680)

7.3.4.3 Adenosine A3 receptors

Another G-protein-coupled adenosine receptor that can negatively modulate adenylyl cyclase activity has been isolated from a rat brain cDNA library. Its binding characteristics with respect to a number of other A1- and A2-selective ligands led Dalziel *et al.*, (1994) to conclude that it was different from existing A1 or A2 receptors, and they proposed the existence of a novel receptor, the A3 receptor. A3 or R226 as it is also known, encodes for an adenosine receptor with non-A1 and non-A2 specificity. Its highest level of expression is found in the testis. The high expression level of the A3 receptor in the testis suggests a possible role for adenosine in reproduction (Zhou *et al.*, 1992).

In humans, the expression of A3 adenosine receptor mRNA is widely distributed with high levels detected in lung and liver, and moderate expression in brain and aorta (Salvatore *et al.*, 1993; Walker *et al.*, 1997).

Synthetic adenosine receptor agonists include 1) CPA which is a highly selective A1 receptor agonist (Collis *et al.*, 1993); CGS21680 specific for A2a receptors, and IB-Meca which is described as a highly potent and selective A3 receptor agonist *in vitro* and *in vivo* (Jacobson *et al.*, 1993; Gallo-Rodriguez *et al.*, 1994).

Little is known about the involvement of A3 receptors in modulating the functions of immune and inflammatory cells, although there is some evidence that activation of this receptor may down-regulate the proinflammatory activities of eosinophils (Walker *et al.*, 1997).

7.3.4.4 Anti-inflammatory activities of A2a receptor agonists

There is indirect evidence for the presence of A1 and A3 receptors on granulocytes (Walker *et al.*, 1997; Bouma *et al.*, 1997; Fredholm *et al.*, 1997) and compelling evidence for the presence of A2a receptors on human neutrophils and lymphocytes (Huang *et al.*, 1997; Varani *et al.*, 1998). The molecular and biochemical mechanisms by which these receptors mediate anti-inflammatory effects on neutrophils and the involvement of cAMP requires clarification.

A2a adenosine receptors in human neutrophils were detected and described using a selective A2a receptor antagonist [^{3}H]-SCH 58261 (Varani *et al.*, 1998). Receptor affinity, as well as the potency of a number of adenosine receptor agonists and antagonists, were determined in receptor binding, adenylyl cyclase activation and superoxide anion production assays. The authors reported that in human neutrophils, [^{3}H]-SCH 58261 directly labels binding sites with pharmacological properties similar to those of A2a adenosine receptors of other tissues. The receptors labelled by [^{3}H]-SCH 58261 were coupled to synthesis of cAMP and inhibition of superoxide anion production in human neutrophils, suggesting that A2a receptor agonists would have cAMP-mediated anti-inflammatory properties.

In another study (Hannon *et al.*, 1998) the adenosine receptor subtype which mediates inhibition of superoxide anion generation induced by FMLP in human neutrophils was investigated using different adenosine receptor agonists. It was found that superoxide anion generation induced by a submaximal concentration of FMLP (1μM) was inhibited in a concentration-dependent manner by adenosine receptor agonists with a rank order of potency of NECA (non-specific receptor agonist)> CGS21680> CPA > 2-Cl-IB-MECA; this order of potency is consistent with the activation of A2a adenosine receptors. The selective A2a receptor antagonist (ZM 241385;100nM) antagonised the inhibition of the oxidative burst induced by each of the agonists (Hannon *et al.*, 1998). These authors also observed that NECA (0.01-10μM) induced a concentration-dependent increase in the intracellular cAMP content of neutrophils. ZM 241385 (0.001-10μM) inhibited this effect in a dose-dependent manner, which is consistent with activation of A2a adenosine receptors. From these results, it was clear that inhibition of the FMLP-induced oxidative burst in human neutrophils by adenosine receptor agonists is mediated via activation of A2a adenosine receptors and is linked positively to cAMP. There was no evidence of A1, A2b, or A3 adenosine receptor-mediated modulation of the oxidative burst.

Using canine cells, Bullough *et al.*, (1995) observed that adenosine, at physiologically relevant concentrations, inhibited the binding of TNFα-activated neutrophils to cardiac myocytes *in vitro*. Adenosine inhibited adhesion of activated neutrophils to cardiac

myocytes with an IC_{50} of 11± 4nM. Inhibition of neutrophil adhesion (92±3% by 100nM adenosine) led to inhibition of myocyte injury (90±6%, as assessed by dye exclusion). The A2 receptor agonist (CGS21680) mimicked adenosine in preventing cell adhesion. These findings were confirmed by Zhao (1996) who reported that CGS21680 inhibited superoxide generation by isolated rabbit neutrophils in a dose-dependent manner as well as neutrophil adherence to the endothelial surfaces of aortic segments.

When the effects of adenosine on a human mast cell line, HMC-1 were investigated *in vitro* by Feoktistov *et al.*, (1995), it was found that the A2a selective agonist, CGS21680 increased cAMP in these cells which was coupled to activation of adenylate cyclase, suggesting that A2a receptors may also down-regulate the pro-inflammatory activities of mast cells.

With respect to other types of immune and inflammatory cells, Huang *et al.*, (1997) incubated mouse splenocytes at $5x10^6$ cells/ml, with or without 20µmol/well CSC (specific A2a receptor antagonist), followed by incubation with different concentrations of CGS21680 (5-20µM) at 37°C for 60 min. As little as 5µM of CGS21680 triggered cAMP accumulation in splenocytes, whereas CSC inhibited this increase. IBMX (cAMP PDE inhibitor) at 50µM/l was included in the systems to prevent catabolism of the accumulated cAMP. To confirm their results, Huang *et al.*, (1997) experimented with two mouse lymphoid organ-derived cell lines, viz. SJL lymphoma and NFS1135 lymphoid cells. These were typed (Northern blot assay) for messenger RNA (mRNA) expression of A2a receptors. It was found that the SJL lymphoid cell line expressed A2a mRNA, whereas the NFS1135 lymphoid cell line had no detectable A2a mRNA. Only A2a mRNA expressing SJL lymphoma cells, but not the A2a mRNA-negative NFS1135 lymphoid cells responded by accumulation of cAMP after incubation with the A2a receptor-specific agonist, CGS21680.

These reports demonstrate that A2a receptors are present on immune and inflammatory cells, including neutrophils, and that A2a receptor agonists have anti-inflammatory potential as a result of their ability to activate adenylyl cyclase and elevate intracellular cAMP.

8. THE AIM OF THE STUDY

The primary aim of my study was to identify the biochemical mechanism of the proinflammatory interactions of the *Pseudomonas aeruginosa* pigment, 1-hydroxyphenazine, with human neutrophils, and to identify anti-inflammatory strategies which neutralize these potentially harmful interactions. To achieve this, the following experiments were performed *in vitro* to measure the effects of 1-hydroxyphenazine (0.3-25μM) on:

1) degranulation (myeloperoxidase and elastase release) by stimulated human neutrophils.

2) calcium fluxes in neutrophils using fura2/AM and radiometric procedures with radiolabelled calcium ($^{45}Ca^{2+}$) to measure alterations in the concentrations of cytosolic Ca^{2+}, as well as to distinguish between net efflux and influx of this cation.

3) intracellular cAMP levels in neutrophils treated with cAMP-elevating agents with or without the pigment. The cAMP-elevating agents used were dibutyryl cAMP (cAMP analogue), phosphodiesterase inhibitors (theophylline, rolipram and GR61170); β_2-adrenoreceptor agonists (salmeterol and salbutamol), and adenosine receptor agonists (CPA, CGS21680 and IB-MECA).

4) elastase release and cellular Ca^{2+} metabolism in the presence or absence of cAMP-elevating agents.

CHAPTER 2

EXPOSURE OF FMLP-ACTIVATED HUMAN NEUTROPHILS TO THE *Pseudomonas aeruginosa*-DERIVED PIGMENT, 1-HYDROXYPHENAZINE (1-HP), IS ASSOCIATED WITH IMPAIRED CALCIUM EFFLUX AND POTENTIATION OF PRIMARY GRANULE ENZYME RELEASE

1. INTRODUCTION

Pyocyanine and 1-hydroxyphenazine (1-HP) are low molecular weight phenazine redox piigments produced by *Pseudomonas aeruginosa* (Ingram *et al.*,1970). Both pigments are present in the sputum of patients iinfected with this microbial pathogen and may contribute to both virulence and persistence by interfering with the mucociliary system (Wilson *et al.*, 1987; Wilson *et al.*, 1988; Munro *et al.*, 1989). Pyocyanine also inhibits epidermal cell growth (Cruickshank *et al.*,1953) and lymphocyte proliferation (Nutman *et al.*, 1987), has antibiotic properties agaiinst other microorganisms (Schoental, 1941) and influences the acquisition of iron by *P aeruginosa* (Cox, 1986). 1-Hydroxyphenazine, but not pyocyanine, potentiates the release of the primary granule enzymes, myeloperoxidase (MPO) and elastase, from activated neutrophils *in vitro* (Ras *et al.*, 1990; Ras *et al.*, 1992). This activity, if it is operative *in vivo*, would favour the development of chronic futile inflammatory responses, resulting in inflammation-mediated tissue damage; this in turn would reduce host defenses and encourage microbial persistence, leading to a self-perpetuating cycle of bacteria-stimulated, host-mediated damage resulting in disease progression (Pier, 1985; Cole *et al.*, 1989).

Although the pro-inflammatory interactions of 1-HP with human neutrophils have been described previously (Ras *et al.*, 1990; Ras *et al.*, 1992), the biochemical mechanisms by which these are achieved have not been elucidated. In the present study, the effects of 1-HP on the stimulus-activated increase in neutrophil cytosolic free Ca^{2+} levels, which precedes, and is also a prerequisite for extracellular release of primary granule enzymes (Knight *et al.*, 1982; Lew *et al.*, 1986; Nüsse *et al.*,1997), have been investigated *in vitro*. In addition, I have measured the levels of cyclic AMP, a second messenger which is intimately involved in the maintanance of Ca^{2+} homeostasis in excitable and non-excitable cells (Schatzmann, 1989; Johannsson *et al.*, 1992), in 1-Hp-treated neutrophils.

2.2 METHODS

2.2.1 *Preparation of the pigment, 1-Hydroxyphenazine (1-HP)*

1-HP was prepared using procedures described in detail by Flood *et al.*, 1972. Briefly, phenazine (500mg) was dissolved in 0.1M HCl (1500ml) and photolyzed by placing the

solution 10cm below an overhead exposed fluorescent light for 3 days. 1-HP was extracted four times in 500ml of chloroform and then from the chloroform layer three times into 1M NaOH (2500ml). The alkaline solution was acidified to pH 1.0 with acetic acid and 1-HP was re-extracted into chloroform. The chloroform layer was washed twice with 6% acetic acid and dried over anhydrous sodium sulfate, and the solvent removed under vacuum. 1-HP was obtained as a single substance as defined by high-pressure liquid chromatography and characterized by ultraviolet spectrophotometry (maximum 273 in 0.1M HCl), gas chromatography-electron impact mass spectrometry, and electrospray mass spectrometry (Watson *et al.*,1986). 1-HP was stable with no loss of activity during incubation or prolonged refrigeration. For the experiments described below, 1-HP was dissolved in dimethyl sulfoxide (DMSO) to give a stock concentration of 10mM and uesd at a final concentration range of 0.3-12.5µM with appropriate DMSO controls (maximum DMSO concentration of 0.125%).

2.2.2 ***Chemicals and reagents***

Unless indicated, all other chemicals and reagents were obtained from the Sigma Chemical Co, St Louis, MO, USA.

2.2.3 ***Preparation of neutrophils***

Human neutrophils were obtained from heparinized (5U preservative-free heparin/ml) (Appendix 4) venous blood of healthy adult volunteers and separated from mononuclear leucocytes by centrifugation on histopaque-1077 (Sigma Diagnostics, St. Louis, MO, USA) cushions at 400g for 25min at room temperature. The resultant cell pellet was suspended in phosphate-buffered saline (PBS) (0.15M (Appendix 3) at pH 7.4 and sedimented with 3% gelatin (Appendix 5) for 15min at 37^{0}C to remove most of the erythrocytes. After centrifugation, residual erythrocytes were removed by selective lysis with 0.83% ammonium chloride (Appendix 1) at 4^{0}C for 10min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%) (Appendix 6), were resuspended to a concentration of 1x10^7/ml in PBS and held on ice until ready for use.

2.2.4 ***Elastase and MPO release***

Neutrophil degranulation was measured according to the extent of release of the

primary granule-derived enzymes, elastase and myeloperoxidase (MPO). Neutrophils were incubated at a concentration of $1x10^7$/ml in indicator-free hank's balanced salt solution (HBSS) with and without 1-HP (0.38-12.5µM) for 10min at 37^0C. The stimulant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1µM), a synthetic chemotactic tripeptide, in combination with cytochalasin B (CB, 1µM) was then added to the cells, which were incubated for 15 min at 37°C. The tubes were then transferred to an ice-bath, followed by centrifugation at 400g for 5min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for elastase and MPO activity using micro-modifications of conventional colorimetric procedures (Paul *et al.*, 1978; Beatty *et al.*, 1982). In the case of elastase, 125µl of supernatant was added to the elastase substrate N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide, (3mM in 0.3% dimethyl sulphoxide DMSO) in 0.05M Tris-HCl (pH 8.0) and elastase activity monitored at a wavelength of 405 nm using a microplate spectrophotometer. In the case of MPO, neutrophil supernatants (20µl) were added to guaiacol and H_2O_2 (final concentrations of 10mM and 5mM respectively) in a final reaction volume of 250µl and enzyme activity monitored spectrophotometrically at 450 nm.

2.2.5 *Spectrofluorimetric measurement of Ca^{2+} fluxes*

Fura-2/AM (Calbiochem Corp., La Jolla, CA, USA) was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments. Neutrophils (1×10^7/ml) were pre-loaded with fura-2 (2 µM) for 30 min at 37°C in phosphate-buffered saline (PBS, 0.15 M, pH 7.4), washed twice and resuspended in indicator-free HBSS containing 1.25 mM $CaCl_2$, referred to hereafter as Ca^{2+}-replete HBSS. The fura-2-loaded cells (2×10^6/ml) were then pre-incubated with 1-HP (0.3-6.25µM) for 10 min at 37°C after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340nm and 500nm respectively. After a stable base-line was obtained (1 min), the neutrophils were activated by addition of FMLP (1µM) and the subsequent increase in fura-2 fluorescence intensity monitored over a 5 min period. The final volume in each cuvette was 3 ml containing a total of 6×10^6 neutrophils. Cytoplasmic Ca^{2+} concentrations were calculated as described previously (Grynkiewicz *et al.*, 1985). Due to non-specific quenching of fluorescence at concentrations of 12.5µM and higher,

6.25µM was the highest concentration of the pigment which could be used with the fura-2 system.

2.2.6 *Radiometric assessment of Ca^{2+} fluxes*

$^{45}Ca^{2+}$ (Calcium-45 chloride, specific activity 18.53 mCi/mg, Du Pont NEN Research Products, Boston, MA, USA) was used as tracer to label the intracellular Ca^{2+} pool and to monitor Ca^{2+} fluxes in resting and activated neutrophils. In the various assays of Ca^{2+} fluxes described below, including those of net efflux and influx, the radiolabeled cation was always used at a fixed, final concentration of 2 µCi/ml, containing 50 nmol cold carrier $CaCl_2$. The final assay volumes were always 5 ml containing a total of 1 x 10^7 neutrophils. The standardisation of the procedures used to load the cells with $^{45}Ca^{2+}$, as well as a comparison with silicone oil-based methods for the separation of labeled neutrophils from unbound isotope, have been described elsewhere (Anderson *et al.*, 1997).

In the first series of experiments, neutrophils (2 x 10^6/ml) were resuspended and equilibrated for 15 min at 37°C in HBSS (final volume 5 ml) containing $^{45}Ca^{2+}$ (2 µCi/ml) as the sole source of Ca^{2+} with and without 1-HP (12.5µM). The amount of cell-associated $^{45}Ca^{2+}$ was then measured immediately prior to, and at 10, 20, 30, 60 and 90 sec, as well as 2, 3 and 5 min after the addition of FMLP (1µM). Reactions were stopped by the addition of 10 ml Ca^{2+}-replete HBSS to the tubes which were transferred to an ice-bath (Anderson *et al.*, 1997). The cells were then pelleted by centrifugatiion at 400 g for 5 min followed by washing with 15 ml ice-cold Ca^{2+}-replete HBSS and the cell pellets finally dissolved in 0.5 ml of triton X-100/0.1 M NaOH and the radioactivity assessed in a liquid scintillation spectrometer. Control, cell-free systems (HBSS and $^{45}Ca^{2+}$ only) were included for each experiment and these values were substracted from relevant neutrophil-containing systems. These results are presented as the amount of cell-associated radiolabeled cation (pmoles $^{45}Ca^{2+}$).

2.2.6.1 *$^{45}Ca^{2+}$-efflux out of FMLP-activated neutrophils*

Neutrophils (1x10^7/ml) were loaded with $^{45}Ca^{2+}$ (2µCi/ml) for 30min at 37^0 C in HBSS, which was free of unlabelled Ca^{2+}. The cells were then pelleted by centrifugation,

washed once with, and resuspended in ice-cold Ca^{2+}-replete HBSS and held on ice until ready for use, which was always within 10min of completion of loading with $^{45}Ca^{2+}$. Using this procedure, the FMLP-activated fura-2 responses of neutrophils, similarly processed in HBSS containing 1µM cold $CaCl_2$, followed by washing with, and suspension in Ca^{2+}-replete HBSS, did not differ from those of cells which had been maintained in Ca^{2+}-replete HBSS throughout (results not shown) indicating that at the time of measurement of efflux in the $^{45}Ca^{2+}$ system, there is no meaningful depletion of intracellular Ca^{2+}. The $^{45}Ca^{2+}$-loaded neutrophils ($2x10^6$/ml) were then pre-incubated for 10min at 37^0C in Ca^{2+} -replete HBSS, in the presence and absence of 1-HP (0.3-12.5µM), followed by activation with FMLP (1µM) and measurement of the kinetics (10,20,30 and 60sec) of efflux of $^{45}Ca^{2+}$. The reactions were terminated by the addition of 10ml ice-cold Ca^{2+}-replete HBSS to the tubes which were transferred to an ice-bath. The cells were then pelleted by centrifugation at 400g for 5min followed by washing with 15ml ice-cold Ca^{2+}-replete HBSS and the cell pellets finally dissolved in 0.5 ml triton X-100/0.1M NaOH and the radioactivity assessed in a liquid scintillation spectrometer. Control, cell-free systems (HBSS and $^{45}Ca^{2+}$ only) were included for each experiment and these values were subtracted from the relevant neutrophil-containing systems.

2.2.6.2 *$^{45}Ca^{2+}$-influx into FMLP-activated neutrophils*

Influx of $^{45}Ca^{2+}$ into neutrophils, uncomplicated by concomitant efflux of the radiolabelled cation, was measured as follows: neutrophils were loaded with cold Ca^{2+}-replete HBSS for 30min at 37^0C after which the cells were pelleted by centrifugation, and then washed once with, and resuspended in ice-cold Ca^{2+}-free HBSS and held on ice until ready for use. Pre-loading with cold Ca^{2+} was undertaken to minimize spontaneous uptake of $^{45}Ca^{2+}$ (unrelated to FMLP activation) in the influx assay. The efficiency of this loading procedure was demonstrated by measurement of the FMLP-activated fura-2 responses of the Ca^{2+}-loaded neutrophils, which were similar to those of neutrophils maintained in Ca^{2+}-replete HBSS (not shown). The Ca^{2+}-loaded neutrophils ($2x10^6$/ml) were then incubated for 10min in the presence and absence of 1-HP (0.3-12.5µM) at 37^0C in Ca^{2+}-free HBSS followed by simultaneous addition of FMLP and $^{45}Ca^{2+}$ (2µCi/ml), or $^{45}Ca^{2+}$ only to control, unstimulated systems. The kinetics of

$^{45}Ca^{2+}$-influx into FMLP-activated neutrophils was then assessed over a period of 5min and compared with those of influx of the radiolabelled cation into the identically-processed, unstimulated cells.

2.2.7 *Radiometric assessment of Na^+ influx*

Neutrophils (2 x 10^6/ml) were suspended in 50 mM Hepes-Tris buffer supplemented with 135 mM choline chloride, 1.1 mM glucose, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM KCl, 1 mM KH_2PO_4 and 100 µM cold NaCl, containing 0.5µCi/ml $^{22}Na^+$ (Sodium-22, specific activity 398.99 mCi/mg, Du Pont NEN Research Products) with and without 1-HP (12.5µM) in a final volume of 5ml at 37°C. Thereafter 100µl of FMLP (1µM final) or an equal volume of buffer was added to each tube and the amount of cell-associated $^{22}Na^+$ measured over a time-course ranging from 10 secs-5 min in control and stimulated cells. Appropriate background values (cells with $^{22}Na^+$ ± FMLP maintained at 4°C throughout the entire time-course of the experiment) were included. Reactions were terminated by the addition of ice-cold PBS, processed as above for $^{45}Ca^{2+}$ efflux/influx experiments and the amount of cell-associated $^{22}Na^+$ determined using a LKB Wallac 1261 Multigamma Counter (Turku, Finland) following lysis of the cells with 0.5 ml of triton X-100/0.1M NaOH.

2.2.8 *Intracellular cAMP levels*

Neutrophils at a concentration of 1x10^7/ml in HBSS were preincubated for 10 min at 37°C with and without 1-HP (12.5µM). Following preincubation, the cells were treated with 1µM FMLP (stimulated cells), or an equal volume of HBSS (resting cells), in a final volume of 1ml, and the reactions terminated and the cAMP extracted by the addition of ice-cold ethanol (65% v/v) at 20 sec, 1 min, 3 min and 5 min after the addition of the stimulant. The resultant precipitates were washed twice with ice-cold ethanol and the supernatants pooled and centrifuged at 2000g for 15 min at 4°C. The supernatants were then transferred to fresh tubes and evaporated at 60°C under a stream of nitrogen. The dried extracts were reconstituted in assay buffer (0.05M acetate buffer, pH 5.8) and assayed for cAMP using the Biotrak cAMP [^{125}I] scintillation proximity assay system (Amersham International plc, Buckinghamsshire, UK), which is a competitive binding radioimmunoassay procedure. The results are expressed as pmoles cAMP/10^7

neutrophils. Because cAMP is rapidly hydrolyzed in neutrophils by phosphodiesterases, these experiments were performed both in the absence and presence of 1μM rolipram (kindly supplied by Dr M Johnson, GlaxoWellcome plc, Stockley Park West, London, UK), a selective type 4 phosphodiesterase inhibitor, the predominant type found in human neutrophils (Torphy, 1998).

2.2.9 *cAMP-dependent Protein Kinase A (PKA) activity*

The effects of 1-HP at 12.5μM on the activity of PKA were measured using the Pierce colorimetric PKA assay kit, Spinzyme™ format (Pierce, Rockford, IL, USA). PKA at 0.5 units of purified catalytic subunit from bovine heart, was coincubated with 1-HP (12.5μM) for 10min at 30°C followed by addition of a synthetic peptide substrate labelled with a fluorescent probe in a final volume of 25μl assay buffer containing 2mM ATP and 100mM cAMP. After incubation at 30°C for 30min, phosphorylated and non-phosphorylated substrate were separated on an affinity membrane which specifically binds the phosphorylated peptide. The membranes were washed, and bound peptide eluted and assayed spectrophotometrically at 570nm.

2.2.10 *Intracellular ATP levels*

These were measured in the lysates of resting and FMLP-stimulated neutrophils in the presence and absence of 1-HP (12.5μM) using a sensitive luciferin-luciferase chemiluminescence procedure (Holmsen *et al.*, 1972). The results are expressed as nmoles ATP/10^7 neutrophils.

2.2.11 *Statistical analysis*

The results of each series of experiments are expressed as the mean values ± SEM. Levels of statistical significance were calculated by Student's *t* test when comparing two groups, or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups. Computer-based software systems Instat II® or Minitab® were used for analyses. Significance levels were taken at a p value of <0.05.

2.3 RESULTS

2.3.1 The effects of 1-HP on elastase and MPO release

The results of these experiments are shown in **Figures 7** and **8**. The results shown in **Figure 7** using cells from 17 different volunteers clearly demonstrated that the release of elastase and MPO from FMLP/CB-activated neutrophils was enhanced in a dose-related manner by pretreatment of cells with 1-HP. For elastase release, statistically significant enhancement was obserrved at concentrations of 0.38µM ($p<0.004$) and upwards, while increased release of MPO by pigment-treated cells achieved statistical significance at concentrations of 1.55µM ($p<0.0002$) and upwards.

This experiment was also performed using neutrophils from a single donor with multiple replicates (10) for control and 1-HP-treated systems (**Figure 8**), and the data analysed by the ANOVA procedure, statistically significant enhancement of release of elastase from 1-HP-treated neutrophils was noted at pigment concentrations of 6.25µM ($p<0.05$) and 12.5µM ($p<0.01$). In the case of MPO, exposure of neutrophils to 3.1, 6.25 and 12.5µM 1-HP resulted in statistically significant ($p<0.05$, 0.001 and 0.001 repectively) dose-related enhancement of this pro-oxidative enzyme following activation of the cells with FMLP:CB. In those experiments using neutrophils from a series of different donors, the magnitude of enhancement of MPO release observed at 12.5µM of 1-HP was slightly less than that at 6.25µM which was probably due to interference with the assay system as a result of the HOCl-scavenging properties of the pigment at concentrations in excess of 6.25µM (Ras *et al.*, 1992). The pigment did not affect the release of either elastase or MPO from unstimulated cells (not shown).

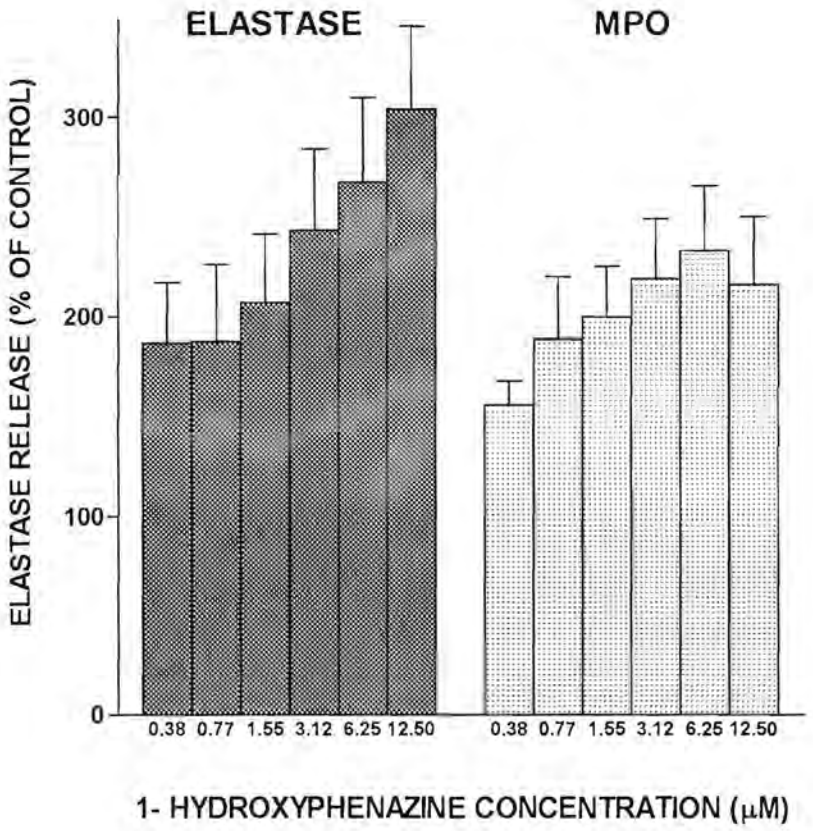


FIGURE 7: Effects of varying concentrations of 1-HP (0.38-12.5μM)on the release of elastase and MPO from FMLP-activated neutrophils. The results of 17 experiments with replicates for control and pigment-treated systems are presented as the mean percentages ± SEMs of the corresponding pigment-free control systems.

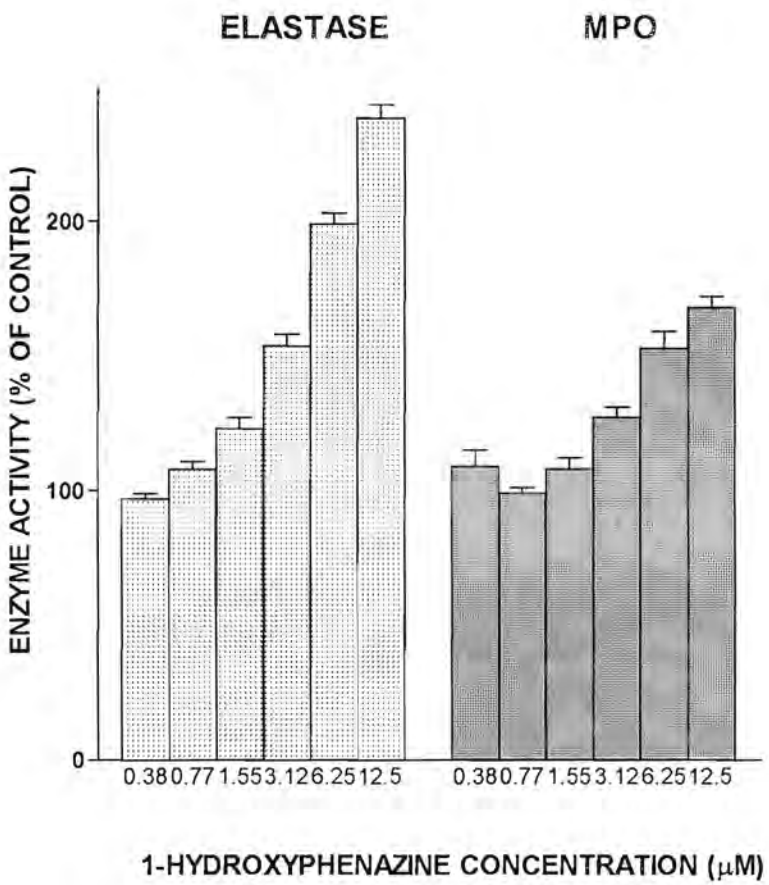


FIGURE 8: Effects of varying concentrations of 1-HP(0.38-12.5μM) on the release of elastase and MPO from FMLP/CB-activated neutrophils. The results of a typical experiment with 10 replicates for control and pigment-treated systems are presented as the mean percentages ± SEMs of the corresponding pigment-free control systems.

2.3.2 Effects of 1-HP on the fura-2 responses of FMLP-activated neutrophils

These results are shown in **Figure 9** and **Table 1.** The results shown in **Figure 9** are traces from 3 typical experiments, which depict the effects of 6.25μM 1-HP on the fura-2 responses of FMLP-activated neutrophils. Addition of FMLP to neutrophils was accompanied by the characteristic, abrupt increase in fura-2 fluorescence due to an increase in the cytosolic concentration of Ca^{2+}. While 1-HP did not affect the abrupt increase in fluorescence intensity, pretreatment of FMLP-activated neutrophils with the pigment retarded the rate of the subsequent decline in fluorescence, indicative of interference with clearance of Ca^{2+} from the cytosol.

The results shown in **Table 1** are those from a larger series of experiments and show peak cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$), as well as the time taken for fluorescence intensity to decline to half peak (t½) values, for neutrophils activated with FMLP in the presence and absence of varying concentrations of 1-HP. As indicated above, 1-HP, at the concentrations used, did not affect the abruptly occurring increase in cytosolic $[Ca^{2+}]i$ following activation of neutrophils with FMLP. However, the pigment at concentrations of 3.1μM and upwards significantly prolonged the time taken for fluorescence to decline to half peak values.

2.3.3 $^{45}Ca^{2+}$ fluxes in activated neutrophils

The time course of $^{45}Ca^{2+}$ fluxes in control and 1-HP (12.5μM)-treated, FMLP-activated neutrophils maintained at 37°C in HBSS containing $^{45}Ca^{2+}$ as the sole source of the cation are shown in **Figure 10**. Following exposure of the control cells to FMLP there was an abrupt decrease in the amount of neutrophil-associated $^{45}Ca^{2+}$ which terminated at about 30 sec and resulted in a mean loss of 33% of the radiolabeled cation. This was followed by an initial slow recovery in the amount of cell-associated $^{45}Ca^{2+}$ (1-2 min) and by accelerated uptake of the cation thereafter (3-5 min) which was completed at 5 min. The amount of $^{45}Ca^{2+}$ released from 1-HP-treated neutrophils 30 sec after the addition of FMLP was significantly less ($p<0.02$) than that released from control neutrophils, while the subsequent rate and extent of uptake appeared similar. However, the apparent decrease in efflux of $^{45}Ca^{2+}$ out of 1-HP-treated cells in the setting of

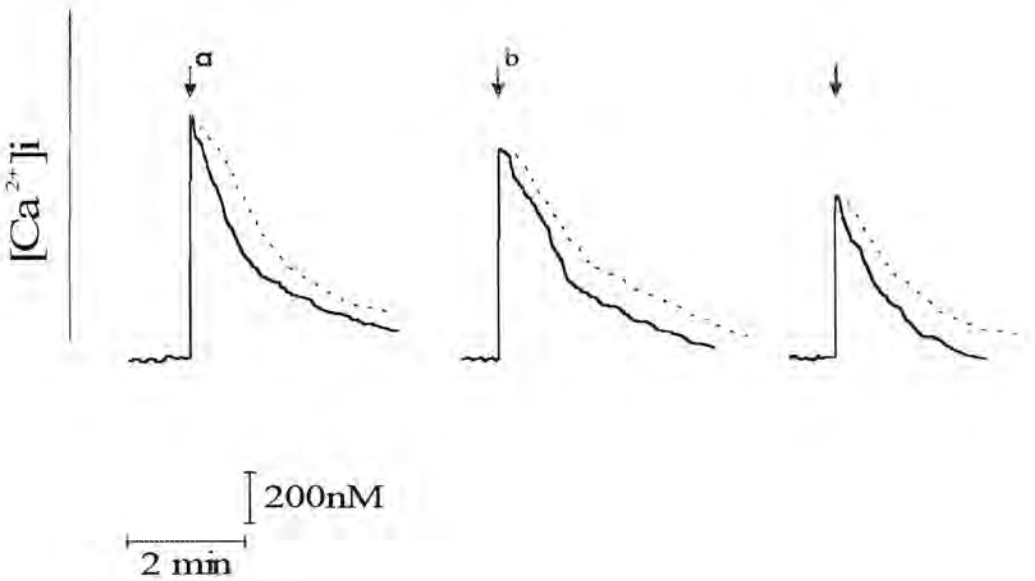


FIGURE 9: FMLP-activated Fura-2 fluorescence responses of control (____) and 1-HP (6.25µM)-treated (- - -) neutrophils. FMLP was added as indicated (↓) when a stable base-line was obtained (± 1 min). The traces shown are from 3 different experiments.

TABLE 1

Peak Cytosolic Calcium Concentrations [Ca^{2+}]i and Time Taken for These to Decline to Half Peak Values in FMLP-Activated Control and 1-HP-Treated Neutrophils

System	Peak [Ca^{2+}]i values (nM)	Time taken to decline to half peak values (min)
FMLP only (control)	849 ± 97	1.10 ± 0.21
FMLP + 1.6 μM 1-hp	848 ± 82	1.14 ± 0.17
FMLP + 3.1 μM 1-hp	837 ± 48	1.40 ± 0.10*
FMLP + 6.25 μM 1-hp	785 ± 75	1.50 ± 0.12*

The results of 10 experiments are expressed as the mean values ± SEMs. The [Ca^{2+}]i value for unstimulated neutrophils was 127 ± 14 nM.

* $p<0.001$ for comparison with the 1-hp-free control system.

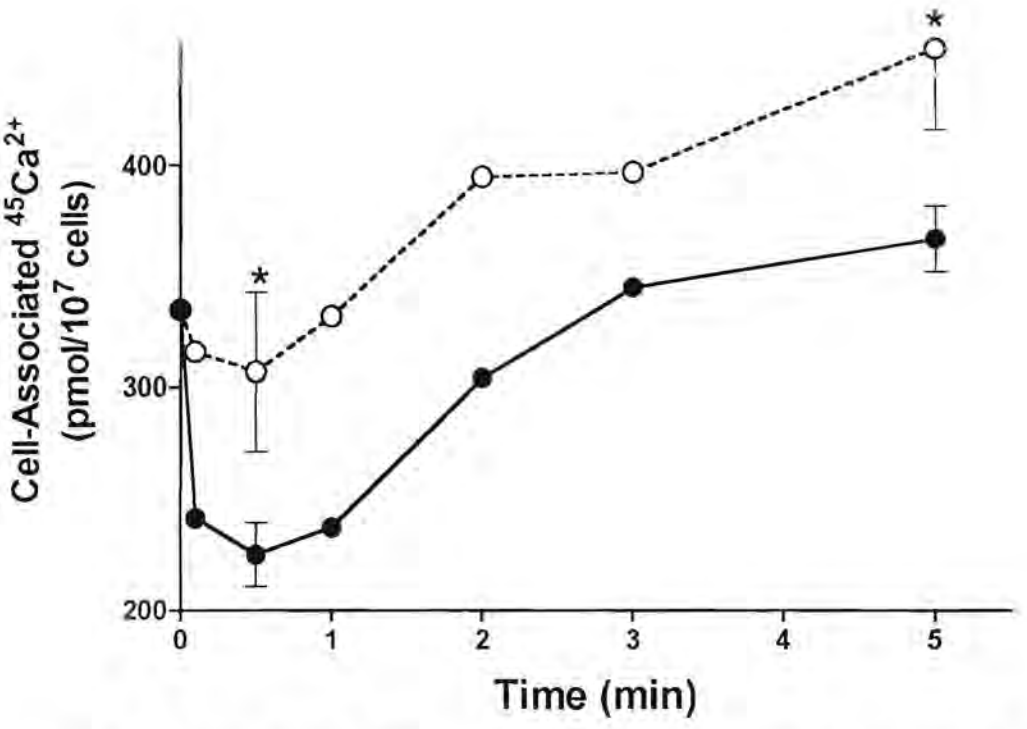


FIGURE 10: Fluxes of $^{45}Ca^{2+}$ following exposure of neutrophils to FMLP in the absence (●-----●) and presence (○- - -○) of 12.5μM 1-HP. The results of 7 different experiments are expressed as the mean amount of cell-associated $^{45}Ca^{2+}$ (pmol/10^7 cells) ± SEMs.

unaffected uptake, resulted in post-stimulation intracellular concentrations of the cation which were higher than those of the pigment-free control cells ($p<0.02$).

2.3.4 Efflux of $^{45}Ca^{2+}$ from FMLP-activated human neutrophils

In these experiments, which were designed to measure net efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils uncomplicated by concomitant influx, cells which had been pre-loaded with $^{45}Ca^{2+}$ and then washed and transferred to Ca^{2+} -replete HBSS (to minimize re-uptake of radiolabelled cation), were activated with FMLP in the presence and absence of 1.6-12.5µM of 1-HP,followed by measurement of the amount of cell-associated $^{45}Ca^{2+}$. The kinetics of efflux of $^{45}Ca^{2+}$ from neutrophils activated with FMLP in the presence and absence of 12.5µM of 1-HP are shown in **Figure 11**. Addition of FMLP to neutrophils resulted in an abrupt efflux of the cation which terminated at about 30sec after the addition of the chemoattractant. Treatment of neutrophils with 1-HP resulted in a statistically significant decrease ($p<0.002$ at 60 secs) in the rate and extent of efflux of $^{45}Ca^{2+}$. The results of a series of experiments in which the effects of varying concentrations of 1-HP on the efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils were investigated using a fixed 60sec incubation period are shown in **Table 2**.

2.3.5 Influx of $^{45}Ca^{2+}$ into FMLP-activated human neutrophils

For these experiments, neutrophils were pre-loaded with cold Ca^{2+} and then transferred to Ca^{2+}-free HBSS prior to activation with FMLP, which was added simultaneously with $^{45}Ca^{2+}$. The results of these experiments, which were designed to measure net influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils in the presence and absence of 1-HP, are shown in **Figure 12.** Activation of control, pigment-free neutrophils with FMLP under these experimental conditions, resulted in a delayed uptake of $^{45}Ca^{2+}$, which occurred after a lag phase of 30-60 sec. Influx of $^{45}Ca^{2+}$ appeared to be a true consequence of activation of neutrophils with FMLP since there was only trivial influx of the radiolabeled cation over the same time-course into control, identically-processed neutrophils, which had not been exposed to FMLP. Pre-treatment of neutrophils with 12.5µM of 1-HP did not detectably alter the extent of influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils.

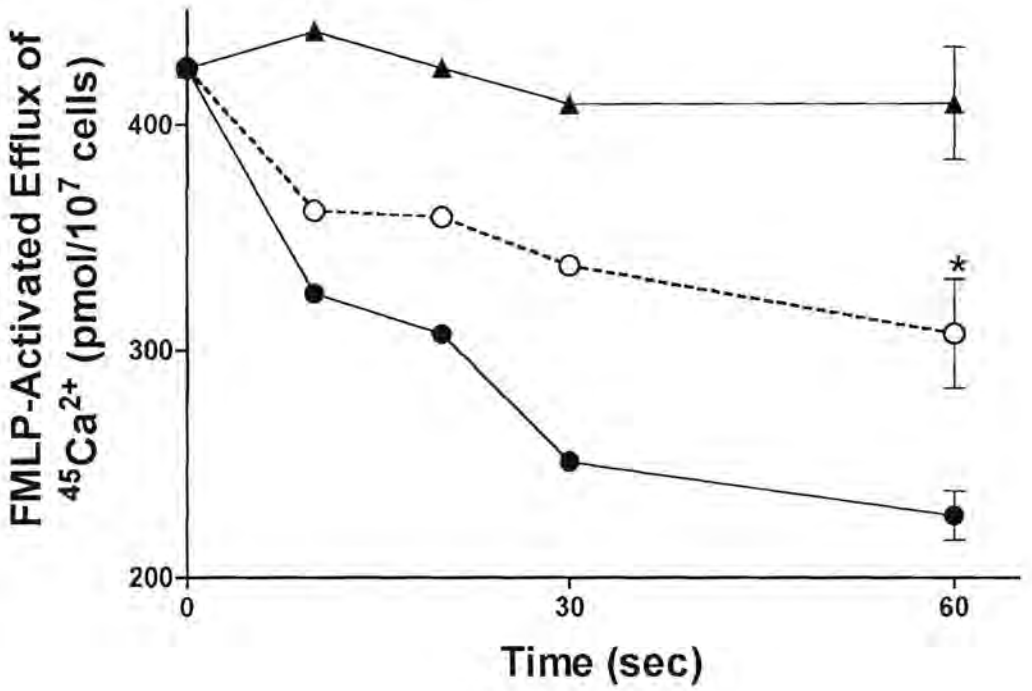


FIGURE 11: Kinetics of efflux of $^{45}Ca^{2+}$ out of resting (▲----▲) and FMLP-activated neutrophils in the absence (●-----●) and presence (○- - -○) of 12.5µM 1-HP. The results of 9 different experiments are expressed as the mean amount of cell-associated $^{45}Ca^{2+}$ (pmol/10^7 cells) ± SEMs.

TABLE 2

The Effects of Varying Concentrations (3.1-12.5 μM) of 1-HP on the Efflux of $^{45}Ca^{2+}$ Out of FMLP-Activated Neutrophils

System	Amount of $^{45}Ca^{2+}$ released from neutrophils 60s after the addition of FMLP (pmol/10^7 cells)
FMLP only	155 ± 10
FMLP + 3.1 μM 1-hp	145 ± 5
FMLP + 6.25 μM 1-hp	120 ± 5*
FMLP + 12.5 μM 1-hp	95 ± 5*

The results of 6 experiments are expressed as the mean values ± SEMs.

* $p<0.02$ - $p<0.0001$ for comparison with the 1-hp-free control system.

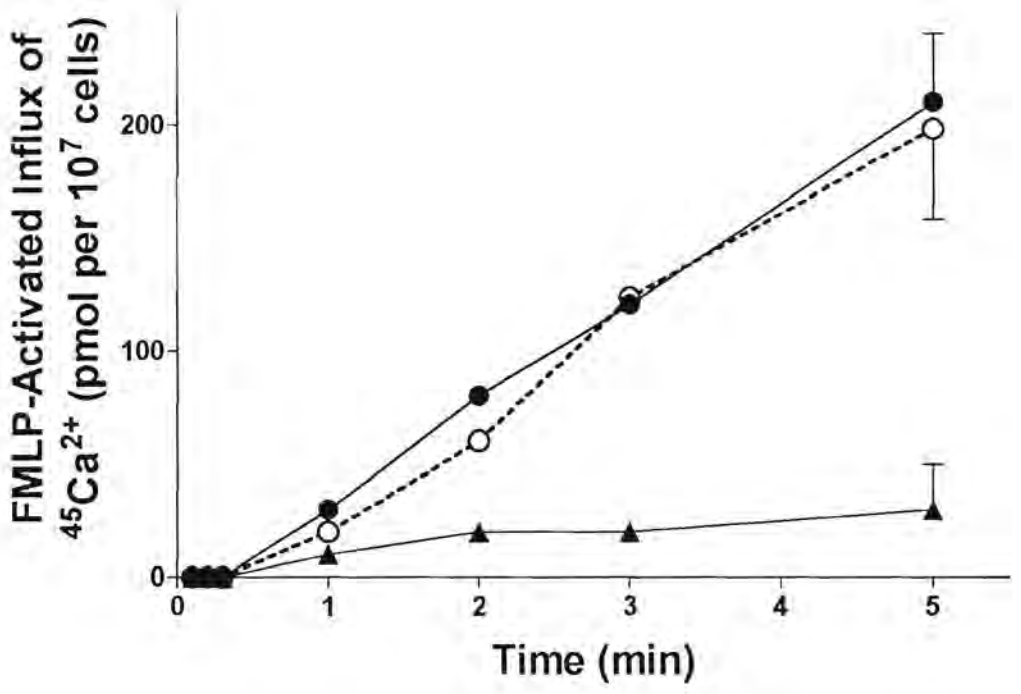


FIGURE 12: Kinetics of influx of $^{45}Ca^{2+}$ into unstimulated (▲------▲) and FMLP-activated neutrophils in the absence (●------●) and presence (○- - -○) of 1-HP (12.5μM). The resutls of 3 different experiments are expressed as the mean uptake of ^{45} Ca^{2+} (pmol/10^7 cells) ± SEMs.

2.3.6 $^{22}Na^{+}$ fluxes in neutrophils

Intracellular concentrations of $^{22}Na^{+}$ were extremely low in unstimulated neutrophils and were unaffected by the addition of FMLP throughout the 5 min time-course of the experiment. At 30 sec and 5 min after the addition of FMLP (times which corresponded with maximum efflux and influx of $^{45}Ca^{2+}$ respectively), the respective amounts of cell-associated $^{22}Na^{+}$ following correction for background values were 178 ± 12 and 144 ± 13 pmol $^{22}Na^{+}/10^{7}$ cells. The corresponding value for unstimulated cells immediately prior to the addition of FMLP was 169 ± 15 pmol $^{22}Na^{+}/10^{7}$ cells (data from 3 separate experiments).

2.3.7 Intracellular cAMP levels

Exposure of resting neutrophils to 1-HP (12.5µM) in the presence or absence of rolipram caused an approximate doubling in intracellular cAMP levels, while those in FMLP-activated cells, although higher than in resting cells, were unaffected by the pigment. One minute after addition of FMLP in the absence of rolipram the respective values for resting neutrophils without and with 1-HP and for FMLP-activated cells without and with the pigment were 14 ± 3, 32 ± 15, 49 ± 11 and 56 ± 28 pmols cAMP/10^{7} cells (data from 4 experiments). The corresponding values for neutrophils treated with rolipram were 64 ± 32, 126 ± 42, 145 ± 50 and 155 ± 64 pmols cAMP/10^{7} cells.

2.3.8 Intracellular ATP levels

Incubation of neutrophils with 12.5µM of 1-HP did not significantly affect the intracellular ATP levels in unstimulated neutrophils. The values for control neutrophils and for cells treated with 1.55, 3.1, 6.25 and 12.5µM 1-HP were 23.9 ± 1.1, 23.8 ± 1.3, 23.1 ± 1.4, 22.8 ± 1.2 and 21.9 ± 1.4 nmoles ATP/10^{7} neutrophils respectively.

2.3.9 cAMP-dependent protein kinase A (PKA)

Coincubation of PKA with 12.5µM 1-HP had no statistically significant effects on the enzyme activity. The activity of PKA coincubated with 1-HP (12.5µM) was 91 ± 4% of the corresponding pigment-free control system. The results were obtained from 5

experiments.

2.4 DISCUSSION

Treatment of human neutrophils with 1-hydroxyphenazine was found to potentiate the release of the primary granule enzymes, elastase and MPO, following exposure of these cells to FMLP. These results, which are essentially confirmatory (Ras *et al.*, 1990; Ras *et al.*, 1992), were obtained using concentrations of the pigment which are well within the range reported to occur in the sputa of cystic fibrosis patients colonized with this intransigent microbial pathogen (Wilson *et al.*,1988). These potentially harmful proinflammatory interactions of 1-HP with neutrophils could not be ascribed to non-specific cytotoxicity, since ATP levels, a sensitive indicator of cellular damage, were similar in both control and pigment-treated cells. This observation indicated that 1-HP may potentiate the biochemical mechanisms which are involved in the activation of neutrophil degranulation, or alternatively, inhibit those which mediate down-regulation of this response. Since degranulation by activated neutrophils is a Ca^{2+}-dependent process (Knight *et al.*, 1982; Lew *et al.*, 1986; Nusse *et al.*, 1997), biochemical processes which mediate increases in the cytosolic concentrations of this cation, as well as those which restore Ca^{2+}-homeostasis, were identified as possible targets of 1-HP.

Data from the Fura-2-based experiments demonstrated that the abruptly-occurring increase in cytosolic Ca^{2+} in FMLP-stimulated neutrophils, a response which is due to the release of the cation from intracellular stores (Anderson *et al.*, 1997; Geiszt *et al.*, 1997), was unaffected by 1-HP. These results indicate that 1-HP does not affect the FMLP/receptor/G-protein interactions which lead to the activation of phospholipase C (Lew *et al.*, 1986), nor does it influence the interactions of inositol triphosphate with Ca^{2+}-mobilizing receptors located on specialized, intracellular cation storage vesicles (Prentki *et al.*, 1984). Although 1-HP did not affect the peak Fura-2 responses of FMLP-activated neutrophils, the rate of decline to basal fluorescence levels was decreased in pigment-treated cells, an observation which is indicative of either a reduction in the efficiency of clearance of cytosolic Ca^{2+}, or enhancement of influx of the cation. To identify which, if any, of these was influenced by 1-HP, radiometric procedures were used to distinguish between net efflux and influx of the cation in the control and in 1-HP-treated, FMLP-activated neutrophils

(Anderson *et al.*, 1997).

Activation of neutrophils equilibrated and maintained in cell-suspending medium containing $^{45}Ca^{2+}$ was accompanied by an abrupt decrease and gradual recovery in cellular $^{45}Ca^{2+}$, events which appeared to correspond to an initial efflux and a delayed influx of the cation. Although the type of radiometric procedure used for this initial series of experiments was unable to distinguish between the net efflux and influx of Ca^{2+}, the results suggested that pretreatment of neutrophils with 1-HP reduces the extent of efflux, without affecting influx of the cation, resulting in intracellular concentrations of Ca^{2+} which are higher than prestimulation values. This observation suggests that exposure of neutrophils to 1-HP not only prolongs the elevation in cytosolic Ca^{2+} in stimulated neutrophils, leading to exaggerated pro-inflammatory activity of these cells, but may also result in Ca^{2+} overload, leading to hyperreactivity of the cells on re-stimulation with Ca^{2+}-mobilizing stimuli.

Exposure of neutrophils, which had been pre-loaded with $^{45}Ca^{2+}$, to FMLP resulted in a rapid efflux of the cation, an observation which is in agreement with previous reports (Gallin *et al.*, 1974; Naccache *et al.*, 1977). Efflux of the cation occurred abruptly, coinciding with the peak increase in cytosolic Ca^{2+}, and terminated about 30seconds after the addtion of FMLP. Treatment of neutrophils with 1-HP resulted in a dose-related reduction in efflux of Ca^{2+} from FMLP-activated neutrophils, indicating interference with plasma membrane cation extrusion systems.

Two types of Ca^{2+} efflux systems have been described in human neutrophils. The first of these is a high capacity, low affinity Na^+/Ca^{2+} exchanger (Simchowitz *et al.*, 1990), the involvement of which is controversial in neutrophils (Volpi *et al.*, 1983; Nasmith *et al.*, 1987; Geiszt *et al.*, 1997). In the present study, I could not demonstrate influx of Na^+ into FMLP-activated neutrophils coincident with efflux of Ca^{2+}. These observations do not support the involvement of a Na^+/Ca^{2+} exchanger in Ca^{2+} efflux in FMLP-activated neutrophils. The second type of Ca^{2+} efflux system is a thapsigargin-insensitive Ca^{2+}-ATPase modulated by calmodulin, which shifts the pump to a higher affinity state for Ca^{2+}, resulting in enhanced maximal velocity (Lagast *et al.*, 1984). This system, which is apparently the major Ca^{2+} efflux system

operative in human neutrophils (Lagast *et al.*, 1984), is the the probable target of 1-HP.

During the brief period of efflux of cytosolic Ca^{2+} out of the FMLP-activated neutrophils, there was no discernible net influx of the cation. Influx was evident only after completion of efflux, being detected at around 30-60seconds, after the addition of FMLP to the cells. As reported previously (Anderson etal., 1997), the observed influx was initially slow, accelerating at around 2-3min and terminating at 5min. This delayed influx of Ca^{2+} into FMLP-activated neuttrophils is characteristic of a store-operated influx, which is operative in many cell types and is necessary for re-filling of the stores (Favre *et al.*, 1996). Treatment of neutrophils with 1-HP did not affect either the rate or the extent of influx of Ca^{2+} into FMLP-activated neutrophils, demonstrating the insensitivity of store-operated influx of the cation to the pigment.

Interestingly, influx of Ca^{2+} into FMLP-activated neutrophils was maximal at a time when Fura-2 fluorescence had subsided to around base-line levels. Although this observation may support the existence of a priviledged store-filling mechanism by which incoming cation bypasses the cytosol (Tsien *et al.*, 1990; Favre *et al.*, 1996), it is more likely to reflect the efficiency of the endo-membrane Ca^{2+}-ATPase, a thapsigargin-sensitive, cAMP-dependent protein kinase (PKA)-modulated cation pump which rapidly sequesters Ca^{2+} into storage vesicles (Schatzmann, 1989; Tao *et al.*, 1992). Several lines of evidence suggest that neither the activation nor the activity of the endo-membrane Ca^{2}-ATPase are influenced by 1-HP. Firstly, the increase in neutrophil cAMP levels which accompanies activation of these cells with FMLP (Anderson *et al.*, 1996; Simchowitz *et al.*, 1980), and which is probably required for activation of the endo-membrane Ca^{2+}-ATPase and down-regulation of the pro-inflammatory activities of activated neutrophils (Anderson *et al.*, 1998), was unaffected by 1-HP, as was the activity of purified PKA in a cell-free assay system. Secondly, although the rate of decline in the Fura-2 fluorescence responses of FMLP-activated neutrophils was slower in 1-HP-treated cells relative to control cells, a return to basal fluorescence was also observed in pigment-treated cells, indicating that the activity of the endo-membrane Ca^{2+}-ATPase was intact.

In conclusion, these observations demonstrate that the *P. aeruginosa*-derived pigment, 1-HP, exerts its pro-inflammatory actions with human neutrophils by acting as an antagonist of the plasma membrane Ca^{2+}-ATPase. Although the exact molecular mechanism of these antagonistic interactions of the pigment with this Ca^{2+}-efflux system remain to be established, the resultant prolongation of the increment in cytosolic Ca^{2+} in activated neutrophils clearly results in hyperactivation of these cells. If operative *in vivo*, these proinflammatory interactions between 1-HP and neutrophils in the bronchial tree are likely to result in "innocent bystander" injury to lung tissue.

CHAPTER 3

THE EFFECTS OF CONVENTIONAL INTRACELLULAR cAMP-ELEVATING AGENTS ON 1-HYDROXYPHENAZINE-MEDIATED INTERFERENCE WITH THE CLEARANCE OF CYTOSOLIC CALCIUM IN, AND ENHANCEMENT OF ELASTASE RELEASE FROM FMLP-ACTIVATED NEUTROPHILS

3.1 INTRODUCTION

It has been reported that receptor-coupled activation of adenylate cyclase and/or inhibition of phosphodiesterases (PDEs) results in increased intracellular concentrations of adenosine 3':5'-cyclic monophosphate (cAMP) and inhibition of the pro-inflammatory activities of neutrophils, eosinophils, monocytes, macrophages, lymphocytes and mast cells (Moore and Willoughby, 1995). Although the exact molecular and biochemical mechanisms of cAMP-mediated anti-inflammatory activity have not been established, the susceptibility of these different cell types suggests that a common pathway involved in cell activation and mediator release may be involved (Anderson *et al.*, 1997).

Since increased concentrations of cytosolic Ca^{2+} precede, and are a pre-requisite, for activation of inflammatory cells, cellular Ca^{2+} metabolism, which is modulated in activated platelets by cAMP-elevating agents (Brace *et al.*, 1985; Johansson *et al.*, 1992; Tao *et al.*, 1992), is a potential target for cyclic nucleotide-mediated modulation. In the case of human neutrophils, exposure of these cells to cAMP-elevating agents has been shown to inhibit the release of Ca^{2+} from intracellular stores (Nielson *et al.*, 1988), or to cause accelerated efflux (De Togni *et al.*, 1984; Villagrasa *et al.*, 1996) and/or decreased influx of the cation (De Togni *et al.*, 1984; Schudt *et al.*, 1991; Ahmed *et al.*, 1995; Villagrasa *et al.*, 1996). Although these data demonstrate that cAMP-elevating agents cause altered Ca^{2+} metabolism in activated neutrophils, the spectrofluorimetric procedures used in all of these studies cannot distinguish between efflux and influx of the cation, which clearly complicates accurate interpretation of the data.

Combination of the fura-2-based spectrofluorimetric method described by Grynkiewicz *et al.*, (1985) and the radioassay method reported by Anderson and Goolam Mahomed, (1997) facilitate distinction between Ca^{2+} efflux and influx. Using these procedures, these authors have demonstrated that the intracellular cAMP-elevating agents rolipram and dibutyryl cAMP accelerate the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils by potentiating the activity of the Ca^{2+} sequestering/resequestering, cAMP-dependent protein kinase-activated

endomembrane Ca^{2+}-ATPase (Anderson *et al.*, 1998). Increased activity of the endo-membrane Ca^{2+}-ATPase, with resultant enhancement of the rate of clearance of Ca^{2+} from the cytosol of activated neutrophils, was exquisitely correlated with the anti-inflammatory activities (inhibition of superoxide production and release of elastase by these cells) of rolipram and dibutyryl cAMP. The plasma membrane Ca^{2+}-ATPase, which removes cytosolic Ca^{2+} by promoting efflux across the plasma membrane, is a cAMP-independent, calmodulin-activated enzyme (Lagast *et al.*, 1984), and consequently, is not positively regulated by cAMP-elevating agents.

The results presented in the previous chapter demonstrate that 1-HP interferes with the activity of the plasma membrane Ca^{2+}-ATPase, resulting in prolongation of intracellular Ca^{2+} transients and enhancement of the release of elastase and MPO in activated neutrophils. I am however, unaware of any pharmacologic agents which could up-regulate the activity of this Ca^{2+}-efflux system as a possible means of overcoming the inhibitory action of 1-HP on restoration of Ca^{2+} homeostasis in activated neutrophils. An alternative strategy, however, may be to use pharmacologic agents which potentiate the activity of the endo-membrane Ca^{2+}-ATPase as a possible means of by-passing 1-HP-mediated inhibition of the plasma membrane Ca^{2+}-ATPase.

In this chapter, the results of a series of experiments are presented, which were designed to investigate the effects of conventional cAMP-elevating agents such as dibutyryl cAMP, selective inhibitors of type 4 PDE (rolipram and GR61170X), a non-selective PDE inhibitor (theophylline) and β_2-adrenoreceptor agonists (salbutamol and salmeterol) on 1-HP-mediated enhancement of release of elastase by FMLP/CB-activated neutrophils, as well as on the fura-2 fluorescence responses of these.

3.2 MATERIALS AND METHODS

The materials and methods used in this chapter are essentially as described in the preceding chapter. The selective PDE 4 inhibitors, rolipram and GR61170X, a recently developed agent, and the β_2-agonists, salbutamol and salmeterol were

kindly provided by Dr Malcolm Johnson of Glaxo-Wellcome plc, Stockley Park West, London, UK. The structure of GR61170X is shown in **Figure 13**. The non-selective PDE-inhibitor, theophylline and the cell-permeable cAMP-analogue, dibutyrylcAMP were purchased from the Sigma Chemical Co. Dibutyryl cAMP was used at 0.05-4mM, salbutamol and salmeterol at 0.1 and 1µM, theophylline at 9-150µM, rolipram at 10-1000nM, and GR61170X, which is twice as potent as rolipram (Dr M. Johnson, personal communication), at 0.01-0.1µM. The concentrations used are those which saturate β_2-adrenoreceptors (salbutamol and salmeterol), or which cause maximal inhibition of PDE (rolipram and GR61170X). 1-HP was used at a fixed concentration of 12.5µM for all the experiments involving elastase release and at 3.1µM for fura-2 fluorescence experiments. For both of these assays, the cAMP-elevating agents were added to the neutrophils during preincubation at 37°C, 5 min before 1-HP, which in turn was added 5 min before FMLP stimulation. All the cAMP-elevating agents were water-soluble with the exception of rolipram and GR61170X. These agents were dissolved in DMSO and appropriate solvent controls were included.

3.3 RESULTS

3.3.1 Effects of the various cAMP-elevating agents on elastase release

The effects of dbcAMP, salbutamol, salmeterol, theophylline, rolipram and GR61170X on the release of elastase from neutrophils activated with FMLP/CB are shown in **Figures 14-18**. DbcAMP, Rolipram, and GR61170X at the concentrations used, caused statistically significant dose-related inhibition of elastase release in the absence of 1-HP and they also antagonized the pigment-mediated enhancement of elastase release from activated neutrophils. Salbutamol, salmeterol and theophylline did not affect the release of elastase from activated neutrophils in the absence of 1-hydroxyphenazine, neither did salbutamol nor salmeterol antagonize 1-HP-mediated enhancement of elastase release from these cells. However, theophylline at concentrations of $\geq$ 38µM showed modest antagonism of 1-HP-mediated enhancement of elastase release from FMLP/CB-activated neutrophils.

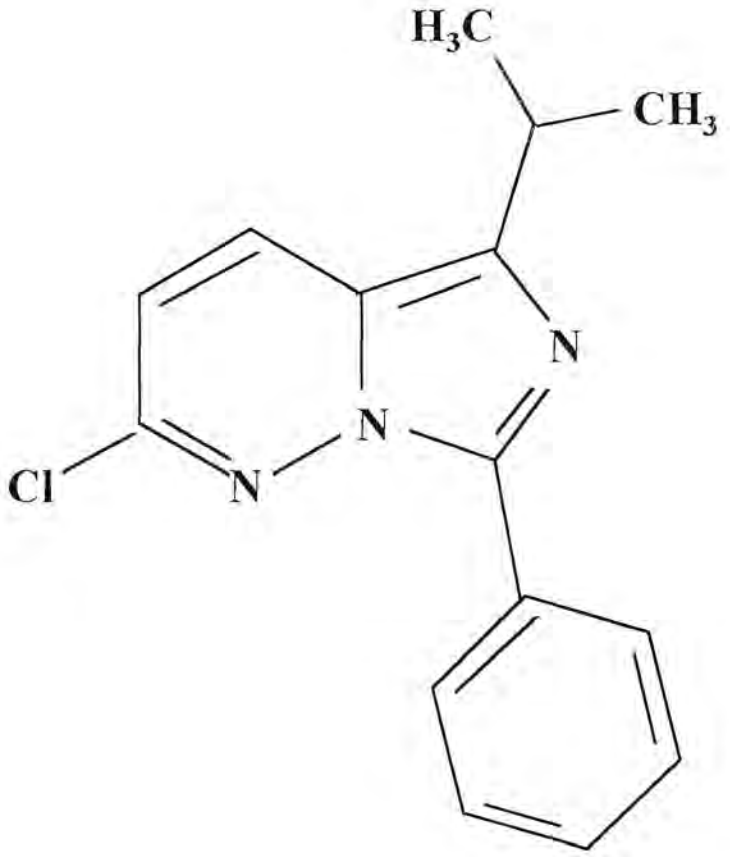


Figure 13: Molecular structure of 2-chloro-5-ispopropyl-7-phenyl-imidazo [1,5-b]pyridazine (GR6117OX)

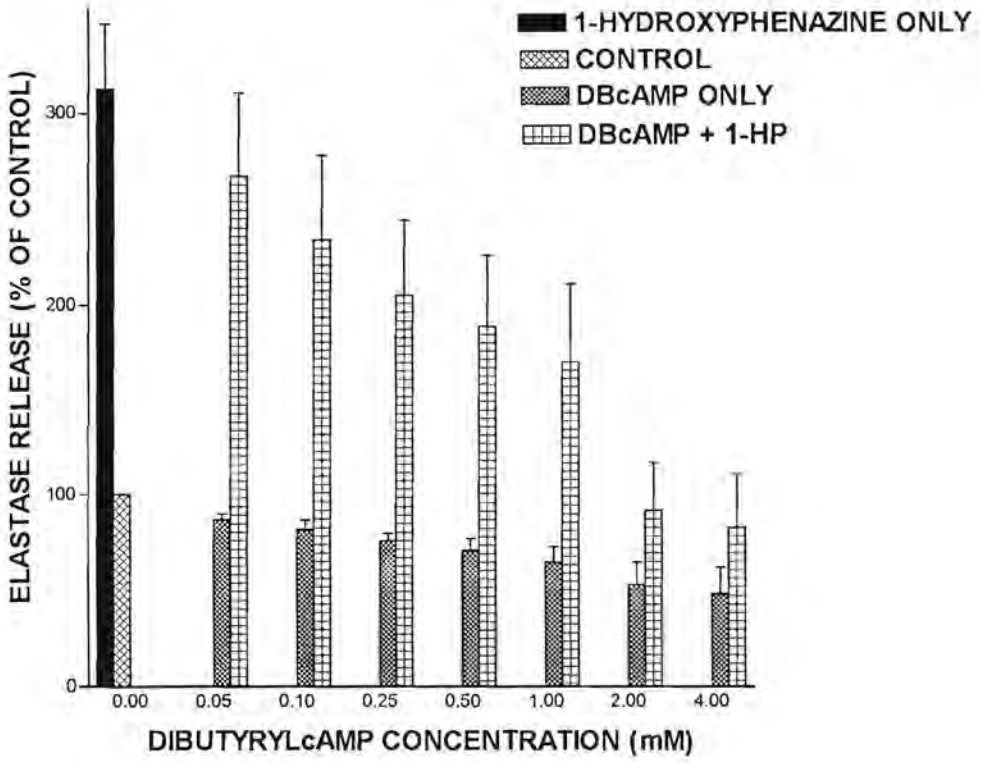


FIGURE 14: Effects of dibutyryl cAMP (0.05-4mM), on the release of elastase from FMLP/CB-activated neutrophils in the presence and absence of 1-HP (12.5μM). The results of 7 different experiments are presented as the mean percentage ± SEMs of the corresponding drug- and pigment-free control systems.

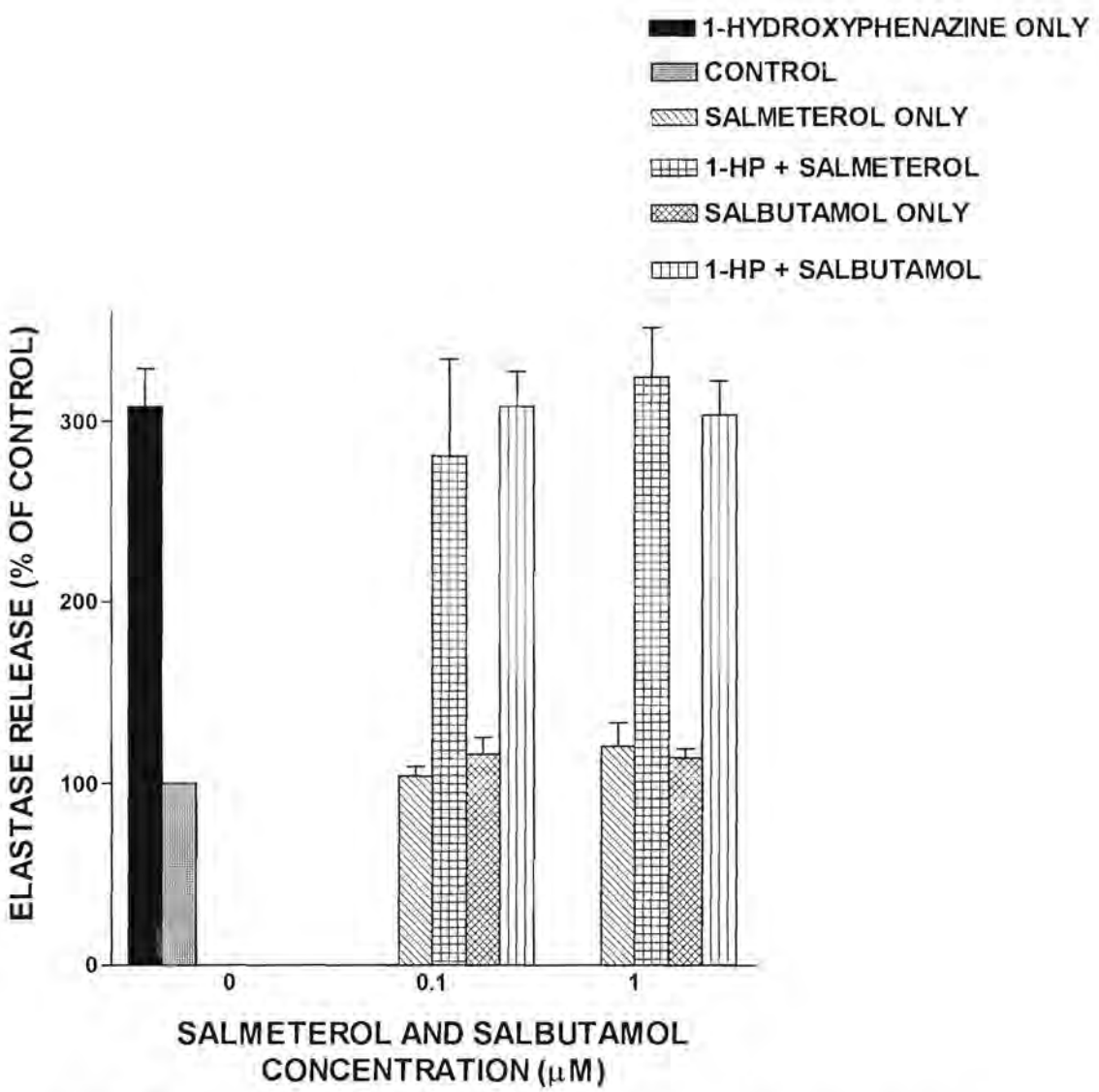


FIGURE 15: Effects of salmeterol and salbutamol (0.1 and 1μM) on the release of elastase from FMLP/CB-activated neutrophils in the presence and absence of 1-HP (12.5μM). The results of 13 different experiments are presented as the mean percentage ± SEMs of the corresponding drug- and pigment-free control systems.

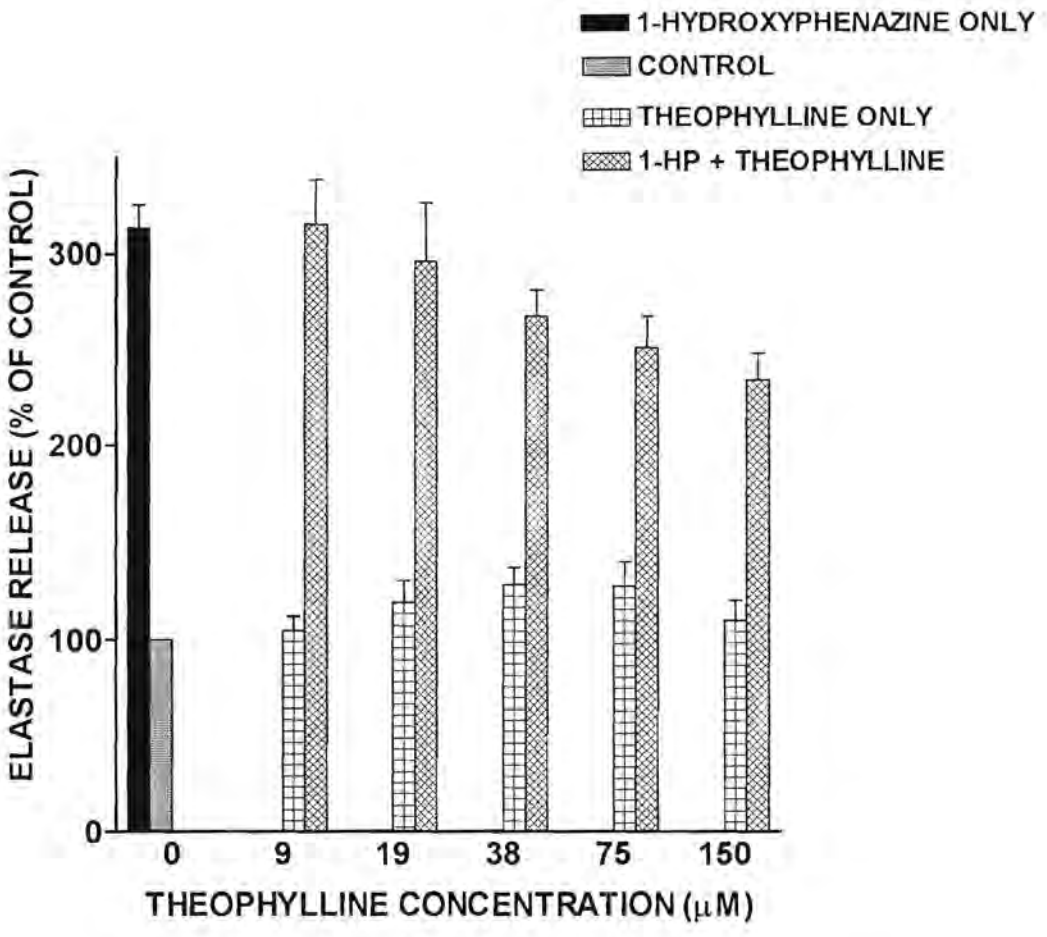


FIGURE 16: Effects of theophylline (9-150μM) on the release of elastase from FMLP/CB-activated neutrophils in the presence and absence of 1-HP (12.5μM). The results of 8 different experiments are presented as the mean percentage ± SEMs of the corresponding drug- and pigment-free control systems.

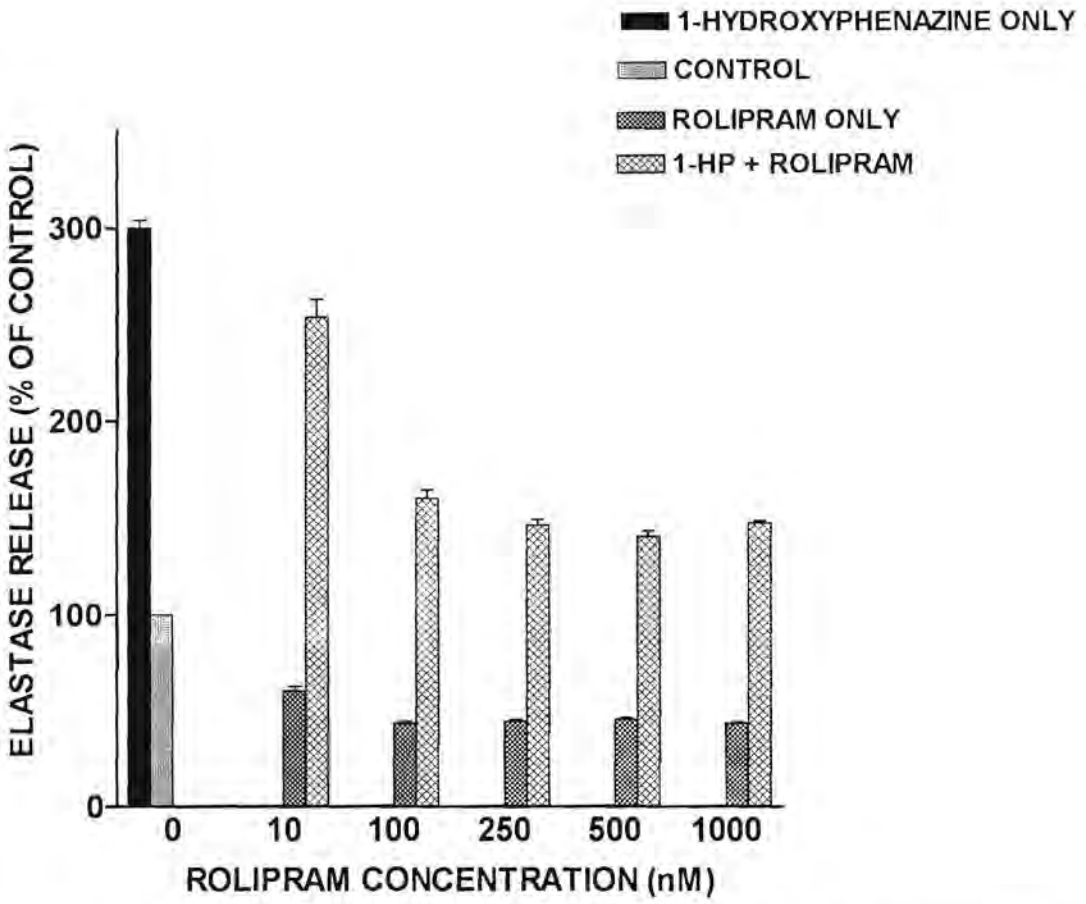


FIGURE 17: Effects of rolipram (10-1000nM) on the release of elastase from FMLP/CB-activated neutrophils in the presence and absence of 1-HP (12.5μM). The results of 8 different experiments are presented as the mean percentage ± SEMs of the corresponding drug- and pigment-free control systems.

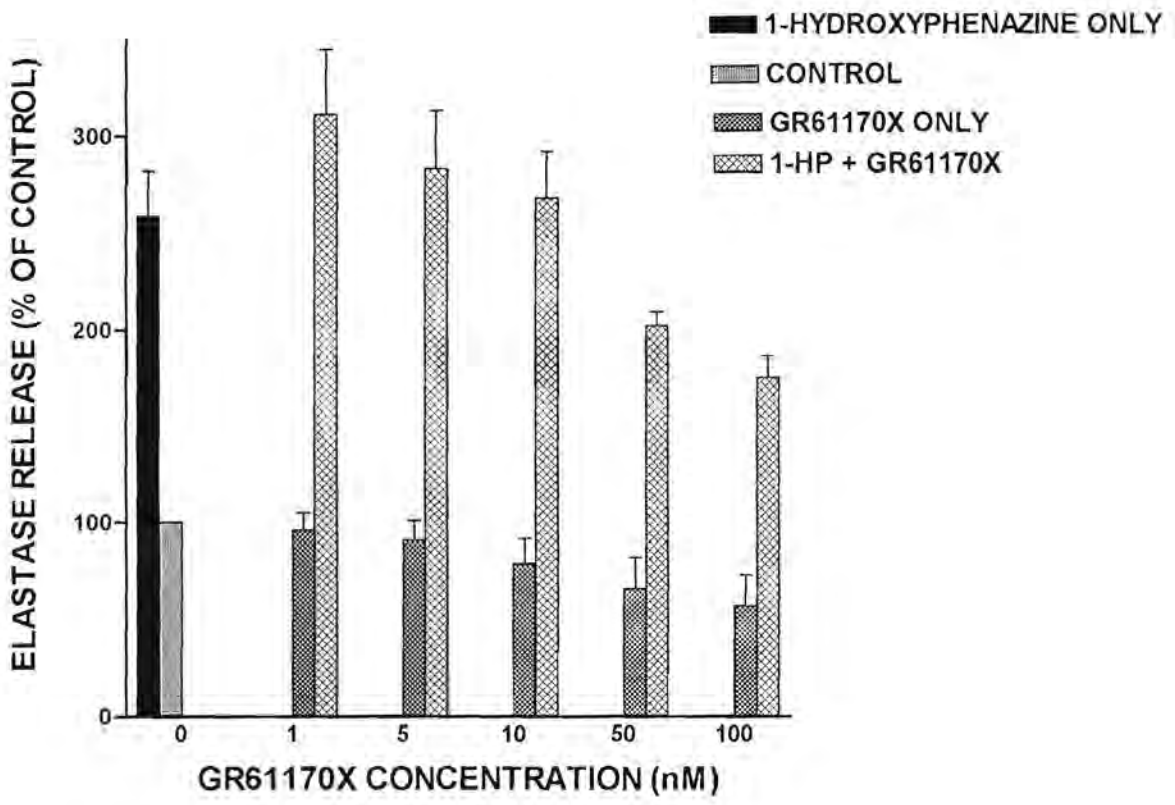


FIGURE 18: Effects of GR61170X (1-100nM) on the release of elastase from FMLP/CB-activated neutrophils in the presence and absence of 1-HP (12.5μM). The results of 4 different experiments are presented as the mean percentage ± SEMs of the corresponding drug- and pigment-free control systems.

3.3.2 **Effects of the cAMP-elevating agents on 1-HP-mediated alterations in the fura-2 responses of FMLP-activated neutrophils**

The results of these experiments are shown in **Figure 19** and **Tables 3** and **4**. The results shown in **Figure 19** are traces of experiments which depict the effects of 1-HP (3.1μM) with and without rolipram (1μM), or dbcAMP (1mM) on the fura-2 responses of FMLP-activated human neutrophils. Addition of dbcAMP or rolipram respectively, did not affect the abrupt increase in peak fluorescence intensity. However, dbcAMP and rolipram hastened the rate of the subsequent decline in fluorescence intensity indicative of accelerated clearance of Ca^{2+} from the cytosol. In contrast to dbcAMP and rolipram, 1-HP retarded the rate of decline in fluorescence intensity as has been shown previously (Anderson *et al.*, 1998). Pretreatment of neutrophils with dbcAMP or rolipram neutralized 1-HP-mediated dysregulation of Ca^{2+} metabolism in FMLP-activated neutrophils.

The results shown in **Table 3** are those from a larger series of experiments and show peak cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$), as well as the time taken for fluorescence intensity to decline to half peak (t½) values, for neutrophils activated with FMLP in the presence and absence of rolipram and dibutyryl cAMP with and without 1-HP (3.1μM). As indicated above, none of the test agents affected the abruptly occurring increase in $[Ca^{2+}]_i$ following activation of neutrophils with FMLP Rolipram and dibutyryl cAMP alone significantly decreased the time taken for fluorescence intensity to decline to half peak values, and also completely neutralized the effects of 1-HP on prolongation of elevated levels of cytosolic Ca^{2+}. Theophylline, salbutamol and salmeterol had either no effects (salbutamol and salmeterol) or minimal effects (theophylline) on the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils and were not tested for their effects on 1-HP-treated neutrophils. GR61170X behaved similarly to rolipram (not shown).The effects of these agents on the FMLP-activated responses of neutrophils in the absence of 1-HP are shown in **Table 4**.

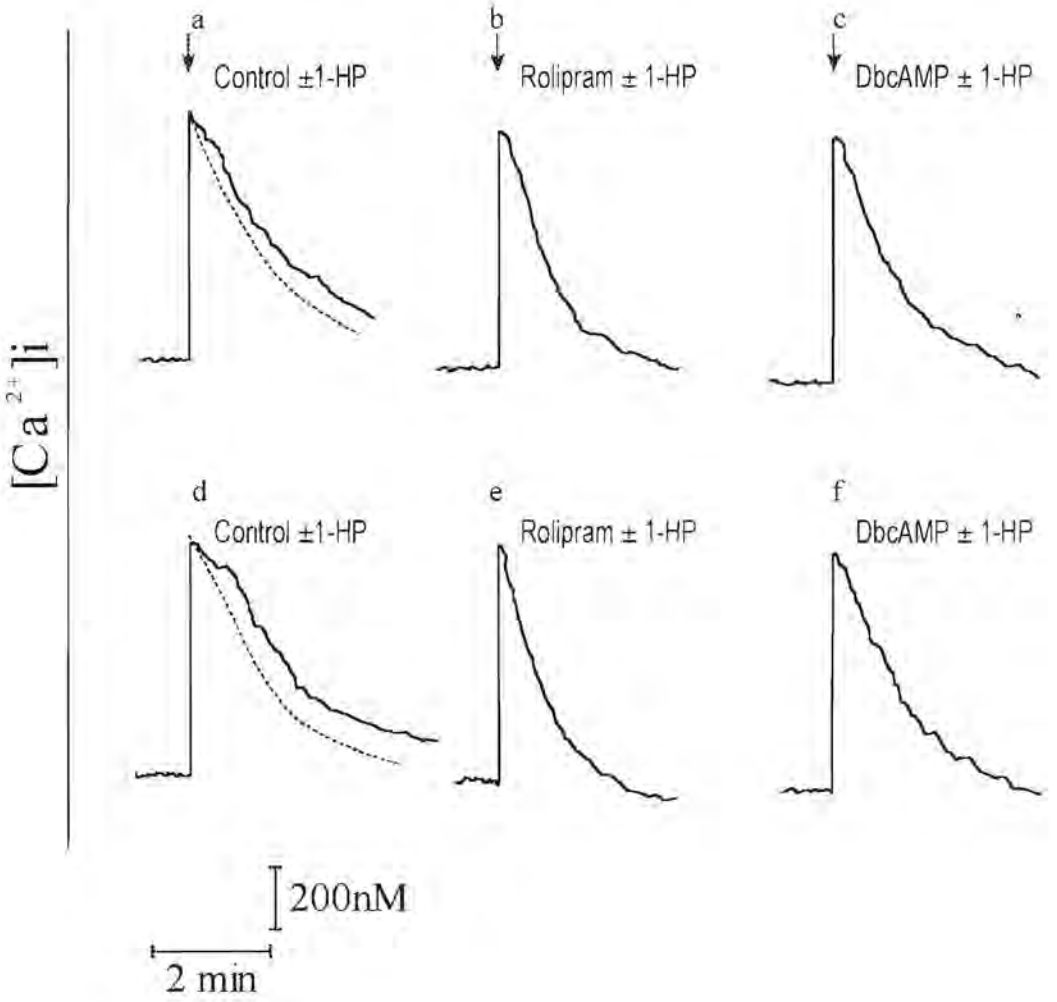


FIGURE 19: Traces from 2 separate experiments (a, b and c for experiment 1 and d, e and f for experiments 2) showing the FMLP-activated fura-2 fluorescence responses of control neutrophils without (-----) or with (——) 1-HP (3.1μM) alone (traces a and d), or with 1-HP in combination with either 1μM rolipram (traces b and e) or 1mM DBC AMP (traces c and f).

TABLE 3:

Peak intracellular calcium concentrations $[Ca^{2+}]_i$ and the time taken for these to decline to half peak values in control and 1-HP-treated neutrophils in the presence and absence of rolipram and dibutyryl cAMP

Agent	Peak $[Ca^{2+}]_i$ values (nM)	Time taken to decline to half peak values (min)
Control	759 ± 44	1.3 ± 0.03
1-HP (3.1µM)	759 ± 28	1.6 ± 0.04
Rolipram (0.1µM) only	773 ± 66	0.73 ± 0.08
Dibutyryl cAMP (1mM) only	773 ± 14	0.90 ± 0.03
1-HP + rolipram	704 ± 33	0.63 ± 0.05
1-HP + dibutyryl cAMP	712 ± 17	0.93 ± 0.05

The results of 4 experiments are expressed as the mean values ± SEMs.

TABLE 4:

Peak intracellular calcium concentrations $[Ca^{2+}]_i$ and time taken for these to decline to half peak values in FMLP-activated neutrophils treated with salbutamol, salmeterol and theophylline

Agent	Peak $[Ca^{2+}]_i$ values (nM)	Time taken to decline to half peak values (min)
Control	759 ± 44	1.3 ± 0.03
Salbutamol (1μM)	726 ± 59	1.25 ± 0.04
Salmeterol (1μM)	742 ± 17.2	1.43 ± 0.03
Theophylline (150μM)	713 ± 48	1.09 ± 0.03

The results of 4 experiments are expressed as the mean values ± SEMs.

3.3.3 **Effects of theophylline, salbutamol and salmeterol on neutrophil cAMP levels**

The effects of rolipram (1μM) on cAMP levels in resting and FMLP-stimulated neutrophils were shown in the previous chapter (chapter 2, page 58), while those of theophylline (150μM), salbutamol (1μM) and salmeterol (1μM) are shown in **Figures 20** and **21**. Theophylline moderately increased cAMP levels in FMLP-activated neutrophils, but not in unstimulated cells, an activity which is probably associated with the ability of this agent to accelerate the clearance of Ca^{2+} from the cytosol of activated neutrophils and to partially antagonize the 1-HP-mediated enhancement of elastase release from these cells. Salbutamol and salmeterol, at concentrations which cause complete occupancy of β_2-adrenoreceptors, had no significant effects on intracellular cAMP levels in either resting or FMLP-stimulated neutrophils, which is in keeping with their inability to accelerate the clearance of cytosolic Ca^{2+} in FMLP-activated neutrophils and to antagonize the stimulatory effects of 1-HP on the release of elastase from FMLP-activated neutrophils. The effects of theophylline, salbutamol and salmeterol on cAMP levels in 1-HP-treated neutrophils were not investigated.

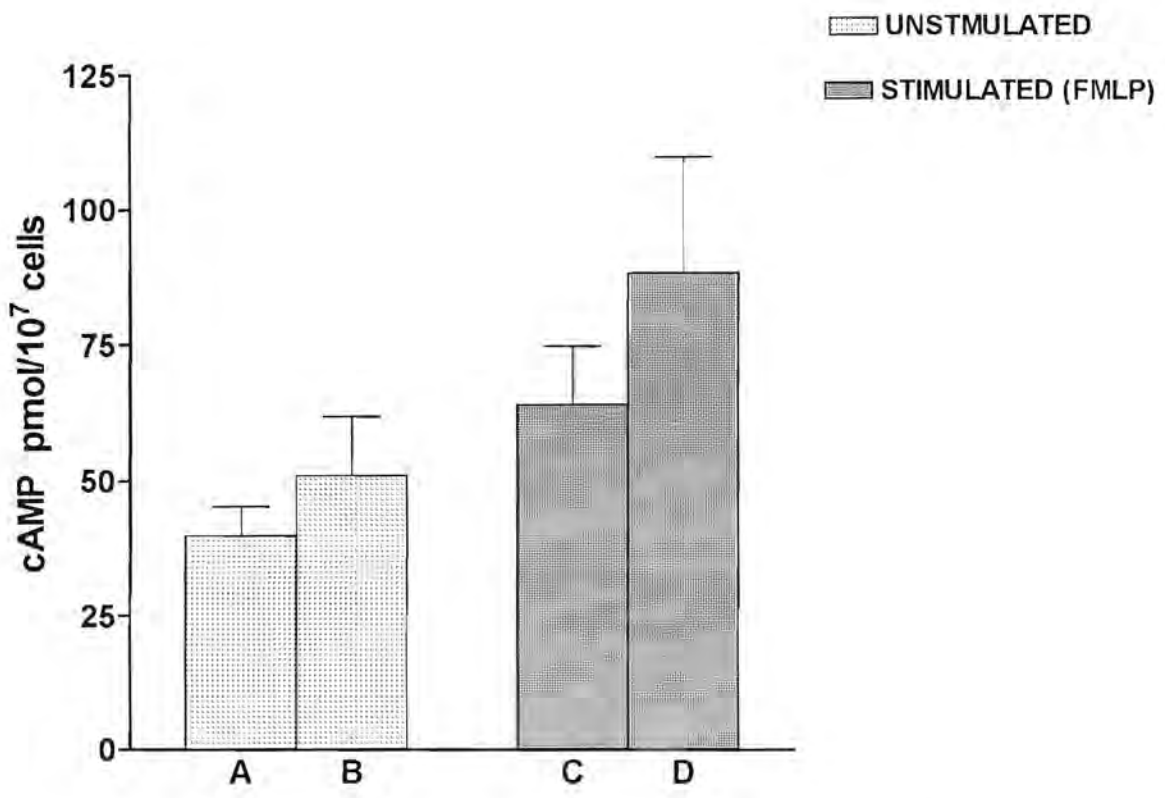


FIGURE 20: Effects of theophylline (150μM) on cAMP levels in unstimulated and FMLP-activated neutrophils. The results of 4 different experiments are presented as the mean cAMP concentrations ± SEMs. A = Unstimulated neutrophils only; B = Unstimulated neutrophils + 150μM theophylline; C = FMLP(1μM)-activated neutrophils; D = FMLP(1μM)-activated neutrophils + 150μM theophylline.

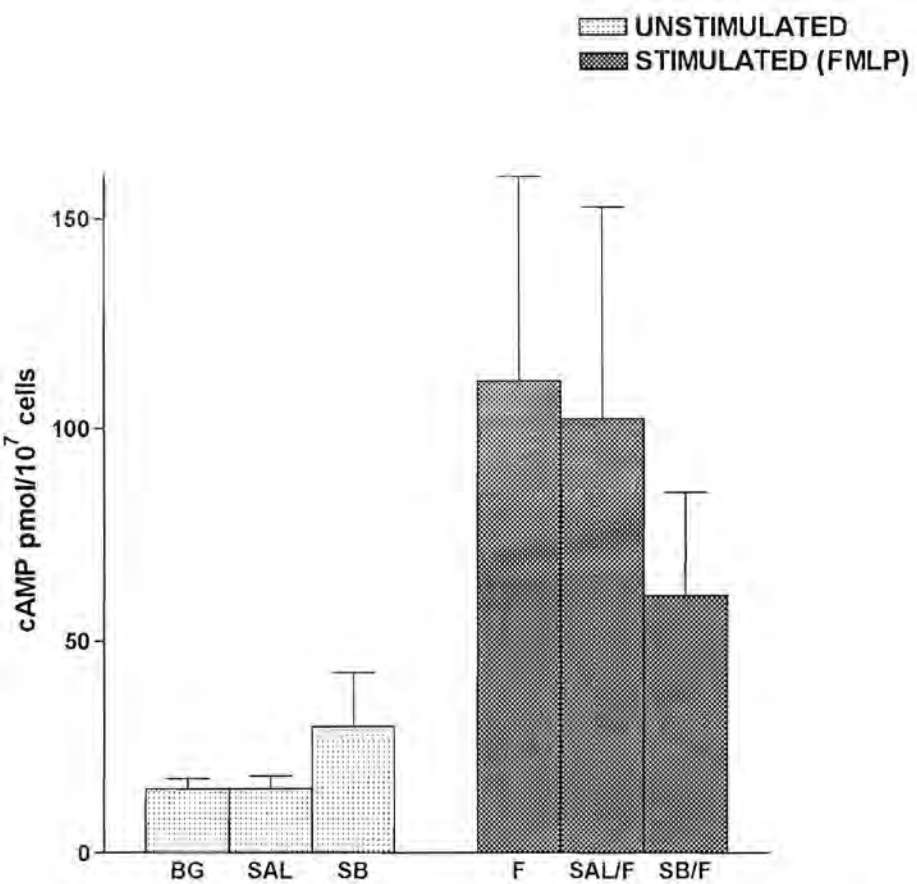


FIGURE 21: Effects of salmeterol (1μM) and salbutamol (1μM) on cAMP levels in unstimulated and FMLP-activated neutrophils. The results of 6 different experiments are presented as the mean cAMP concentrations ± SEMs. BG = Background; SAL = Salmeterol; SB = Salbutamol; F = FMLP.

DISCUSSION

Oxidant generation and degranulation by activated neutrophils are sensitive to inhibition by cAMP-elevating agents especially when the stimuli used cause mobilization of Ca^{2+} (Ladd *et al.*, 1985; Nielson *et al.*, 1990; Moore and Willoughby, 1995; Anderson *et al.*, 1998). In my study, dbcAMP, rolipram and GR61170X (a new selective type 4 phosphodiesterase inhibitor), caused statistically significant dose-related inhibition of elastase release by FMLP/CB-activated neutrophils. These same agents, and to a lesser extent theophylline, also caused dose-related antagonism of the stimulatory effects of 1-HP on the release of elastase from activated neutrophils. However, the two β_2-adrenoreceptor agonists, salmeterol and salbutamol, did not affect elastase release from FMLP/CB-activated neutrophils in either the presence or absence of 1-HP.

Although none of the cAMP-modulating agents used affected the release of Ca^{2+} from intracellular storage pools, the rate of decline of the peak fura-2 fluorescence in FMLP-activated neutrophils was hastened by dbcAMP and rolipram, and to a lesser extent by theophylline, but not by salmeterol or salbutamol. This observation is indicative of the accelerated clearance of Ca^{2+} from the cytosol by cAMP-elevating agents. Anderson *et al.*,(1998) showed that relative to rolipram and dcAMP, the effects of theophylline on the rate of decline of peak fura-2 fluorescence were delayed and less impressive. Nielson *et al.*, (1987) and Anderson *et al.*, (1998) also showed that salbutamol only inhibited the pro-inflammatory activities of activated neutrophils when used in combination with rolipram. Interestingly, pretreatment of neutrophils with either dbcAMP or rolipram completely antagonized the 1-HP-mediated prolongation of intracellular Ca^{2+} transients, an effect which may explain the ability of these agents to neutralise the pigment-induced enhancement of the release of elastase from these cells.

Exposure of resting neutrophils to 1-HP in the presence or absence of rolipram caused an approximate doubling in intracellular cAMP levels, while those in FMLP-activated cells, although higher than in resting cells, were unaffected by the pigment. Salbutamol and salmeterol, at concentrations which cause complete occupancy of

β_2-adrenoreceptors, had no significant effects on intracellular cAMP levels in either resting or FMLP-stimulated neutrophils. This is in keeping with their inability to accelerate the clearance of cytosolic Ca^{2+} in FMLP-activated neutrophils and to antagonize the stimulatory effects of 1-HP on the release of elastase from FMLP-activated neutrophils. On the other hand, theophylline moderately increased cAMP levels in FMLP-activated neutrophils, but not in unstimulated cells, an activity which is probably associated with the ability of this agent to accelerate, albeit weakly, the clearance of Ca^{2+} from the cytosol of activated neutrophils and to partially antagonize the 1-HP-mediated enhancement of elastase release from these cells.

I have shown earlier that 1-HP interferes with the activity of the plasma membrane Ca^{2+}-ATPase, resulting in prolongation of intracellular Ca^{2+} transients in, as well as enhancement of elastase release from activated neutrophils. The cAMP-elevating agents, dbcAMP, rolipram and the new selective PDE type 4, GR61170X, were able to neutralize 1-HP-mediated interference with the clearance of cytosolic Ca^{2+} and enhancement of elastase release from activated neutrophils. The accelerated clearance of cytosolic Ca^{2+} by these agents was probably achieved by upregulation of the activity of the endomembrane Ca^{2+}-ATPase, resulting in enhancement of resequestration of the cation.

Type 4 PDE inhibitors may benefit patients colonized with *Pseudomonas aeruginosa,* since they neutralize the pro-inflammatory effects of 1-hydroxyphenazine. Second generation type 4 PDE inhibitors, which appear to have an improved therapeutic window, may be particularly useful in this respect (Torphy, 1998; Underwood *et al.*, 1998).

CHAPTER 4

INVESTIGATION OF THE EFFECTS OF ADENOSINE RECEPTOR AGONISTS ON 1-HYDROXYPHENAZINE-MEDIATED ENHANCEMENT OF RELEASE OF ELASTASE FROM ACTIVATED NEUTROPHILS AND ITS RELATIONSHIP TO ALTERATIONS IN INTRACELLULAR cAMP LEVELS

4.1 INTRODUCTION

Adenosine is a broad-spectrum physiologic anti-inflammatory agent released by many cell types. The anti-inflammatory and other physiologic effects of adenosine are achieved through the interactions of this agent with at least four different types of 7-transmembrane, G-protein/adenylate cyclase-coupled adenosine receptors known as A1, A2a, A2b and A3 (Cronstein, 1994). Interestingly, sustained elevations in circulating adenosine concentrations leading to uncontrolled interactions with A2a receptors on T- and B-lymphocytes is thought to underpin the hyporesponsiveness of these cells in patients with the adenosine deaminase deficiency variant of severe combined immunodeficiency disease (SCID). The anti-inflammatory and immunosuppressive potential of adenosine is, however, restricted by two factors, *viz.* the short half-life (2 seconds) in the circulation of this agent, as well as receptor promiscuity. Both of these limitations have been overcome by the development of selective pharmacologic adenosine receptor agonists (Cronstein, 1994).

There is indirect evidence for the presence of A1 and A3 receptors on granulocytes (Walker *et al.*, 1997; Bourma *et al.*, 1997; Fredholm *et al.*, 1997) and fairly convincing evidence for the presence of A2a receptors on human neutrophils (Varani *et al.*, 1998). However, the exact involvement of these receptors in mediating the anti-inflammatory effects of adenosine remains to be conclusively established (Bouma *et al.*, 1997; Fredholm *et al.*, 1997), as does the role of cAMP (Cronstein, 1994; Varani *et al.*, 1998).

In the present study, the major objectives were to investigate the effects of adenosine receptor agonists (A1, A2a, and A3) on the proinflammatory activity (elastase release) of 1-HP-treated and untreated neutrophils, as well as the effects of these agents on intracellular cAMP. Receptor specificity was evaluated using ZM241385, a highly selective antagonist of the A2a receptor type (Poucher *et al.*, 1995). These investigations were undertaken to establish the potential of adenosine receptor agonists to circumvent the proinflammatory effects of 1-HP on neutrophils and to identify the specific receptors involved.

4.2 MATERIALS AND METHODS

Materials and methods are the same as those described in chapter 2. In the present study however, the test pharmacologic agents used were the adenosine receptor agonists CPA (A1-receptor agonist), CGS21680 (A2a receptor agonist) and IB-MECA (A3 receptor agonist). These agents were kindly supplied by Dr Malcolm Johnson of Glaxo-Wellcome, UK. ZM241385 was purchased from Tocris Cookson Ltd, UK. CPA, CGS21680 and IB-MECA all of which are water-soluble, were used at concentrations of 0.01-1μM for elastase release assays and at 1μM in assays of intracellular cAMP. Neutrophils were incubated at a concentration of 1 x 10^7/ml in HBSS in the presence or absence of either CPA, CGS21680 or IB-MECA, with or without 1-HP at a fixed, final concentration of 12.5μM in the presence or absence of ZM241385 (2.5μM) for 10min at 37°C. 1-HP was added to the cells 5 min after the various adenosine receptor agonists. When it was used, ZM241385 was added to the cells 1 min before the various adenosine receptor agonists. The stimulant FMLP (1μM) in combination with cytochalasin B (0.5μg/ml) was then added and the reaction mixtures incubated for a further 15 min at 37°C. The tubes were then transferred to an ice bath, followed by centrifugation at 400 g for 5 min to pellet the cells. Thereafter, the procedures were identical to those described in chapter 2.

With respect to cAMP, neutrophils at a concentration of 1 x 10^7/ml in HBSS were preincubated for 10 min at 37°C with either CPA, CGS21680 or IB-MECA, all at a concentration of 1μM in the presence or absence of 1-HP (12.5μM) which was added 5 min after the adenosine receptor agonists. Following preincubation, the cells were activated with 1μM FMLP and the procedures thereafter were described in chapter 2.

Measurement of fluctuations in the concentrations of cytosolic Ca^{2+} during exposure of neutrophils to the various adenosine receptor agonists were not performed in this study since these form the basis of an ongoing study by a colleague (Dr Z. Visser). However, with her permission, these are alluded to in the discussion as a personal communication.

4.3 RESULTS

4.3.1 Effects of the adenosine receptor agonists on elastase release from FMLP/CB-activated neutrophils.

The effects of CPA, CGS21680 and IB-MECA on the release of elastase from neutrophils activated with FMLP/CB are shown in **Figures 22-24**. CPA did not affect the release of elastase from activated neutrophils in the absence of 1-HP, nor did it antagonize the pigment-mediated enhancement of release of this protease. Both CGS21680 and IB-MECA caused a dose-dependent inhibition of release of elastase in the absence of 1-HP and also antagonized the pigment-mediated enhancement of release of elastase by activated neutrophils.

4.3.2 Effects of the A2a adenosine receptor antagonist (ZM241385) on CGS21680 and IB-MECA-mediated interference with 1-HP-induced enhancement of elastase release

The effects of ZM241385(2.5μM) alone as well as on CGS21680 and IB-MECA-(both
at 1μM) mediated inhibition of elastase release by 1-HP(12.5μM)-treated neutrophils and pigment-free, control neutrophils are shown in **Figures 25** and **26**. Pretreatment of neutrophils with ZM241385 *per se* caused an increase in elastase release following activation of the cells with FMLP/CB which was of similar magnitude to that caused by 1-HP. Treatment of neutrophils with this agent (ZM241385) also antagonized the inhibitory effects of both CGS21680 and IB-MECA on degranulation in both the absence and presence of 1-HP.

4.3.3 Effects of CPA, CGS21680 and IB-MECA on neutrophil cAMP levels

The results of these experiments which are depicted in **Figures 27 and 28** show the effects of the adenosine receptor agonists on cAMP in resting and stimulated neutrophils in the presence or absence of 1-HP. CPA alone did not affect cAMP levels in either control or 1-HP-treated neutrophils (results not shown). However, treatment of neutrophils with IB-MECA and CGS21680 resulted in increased cAMP levels in both resting and FMLP-activated neutrophils in the case of IB-MECA, and in stimulated neutrophils in the case of CGS21680. These increases were of similar magnitude in both pigment-free and 1-HP-treated neutrophils

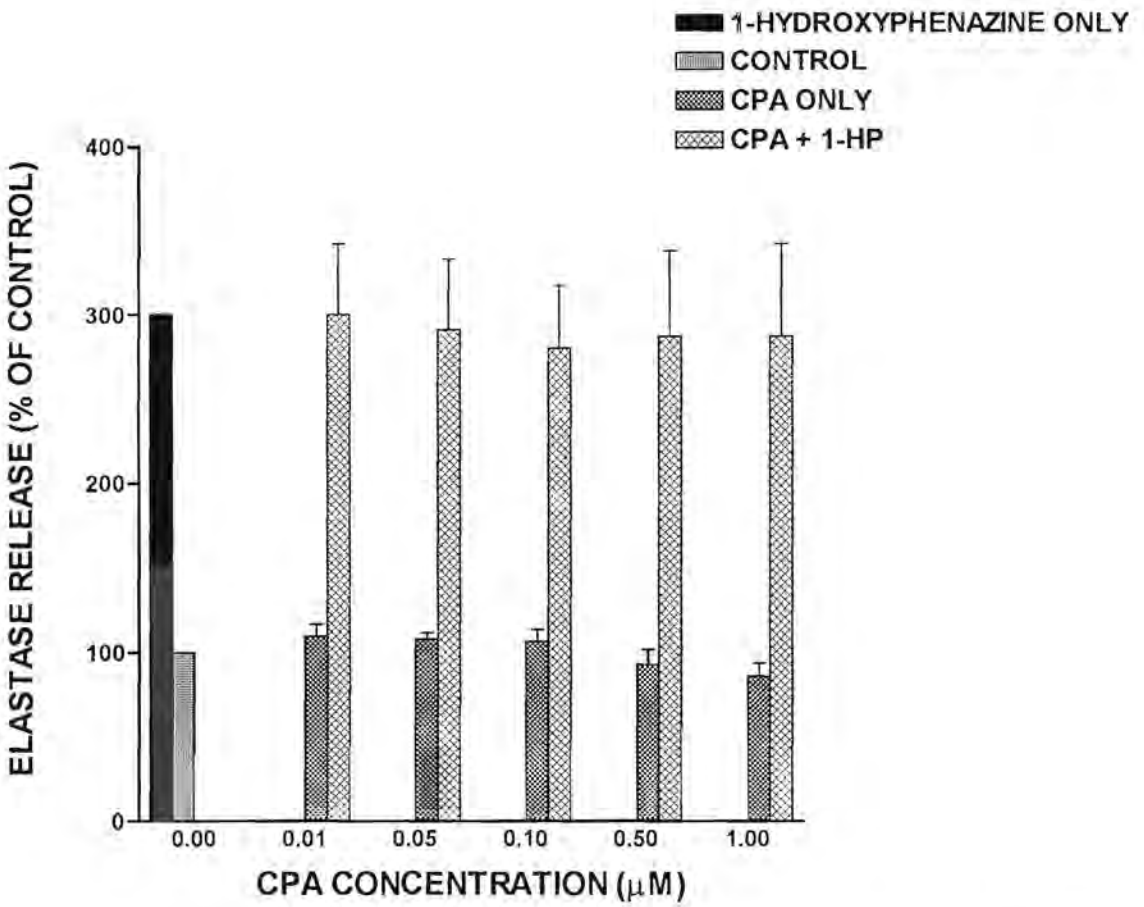


FIGURE 22: Effects of the adenosine A1 receptor agonist, CPA (0.01-1μM) on the release of elastase from FMLP/CB-activated neutrophils in the presence and absence of 1-HP (12.5μM). The results of 4 different experiments are expressed as the mean percentage ± SEMs of the corresponding drug- and pigment-free control systems.

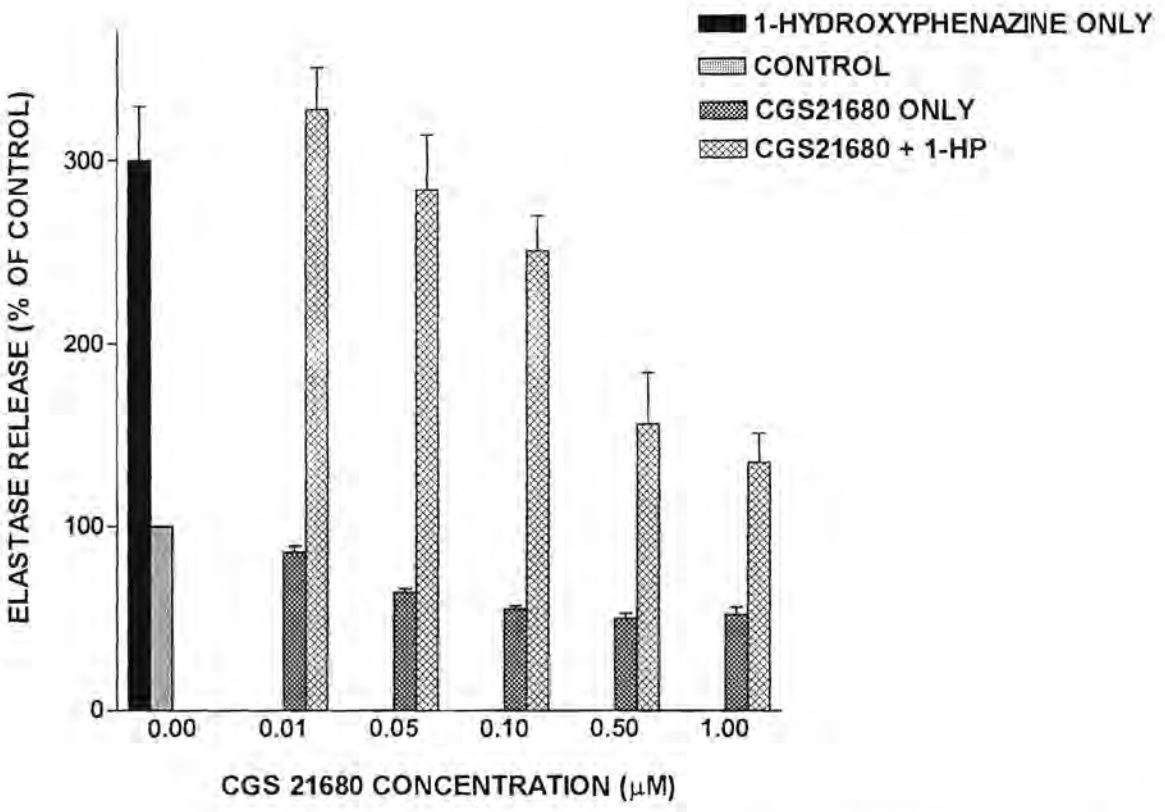


FIGURE 23: Effects of the A2a adenosine receptor agonist, CGS21680 (0.01-1μM) on the release of elastase from FMLP/CB-activated neutrophils in the presence and absence of 1-HP (12.5μM). The results of 4 different experiments are presented as the mean percentage ± SEMs of the corresponding drug- and pigment-free control systems.

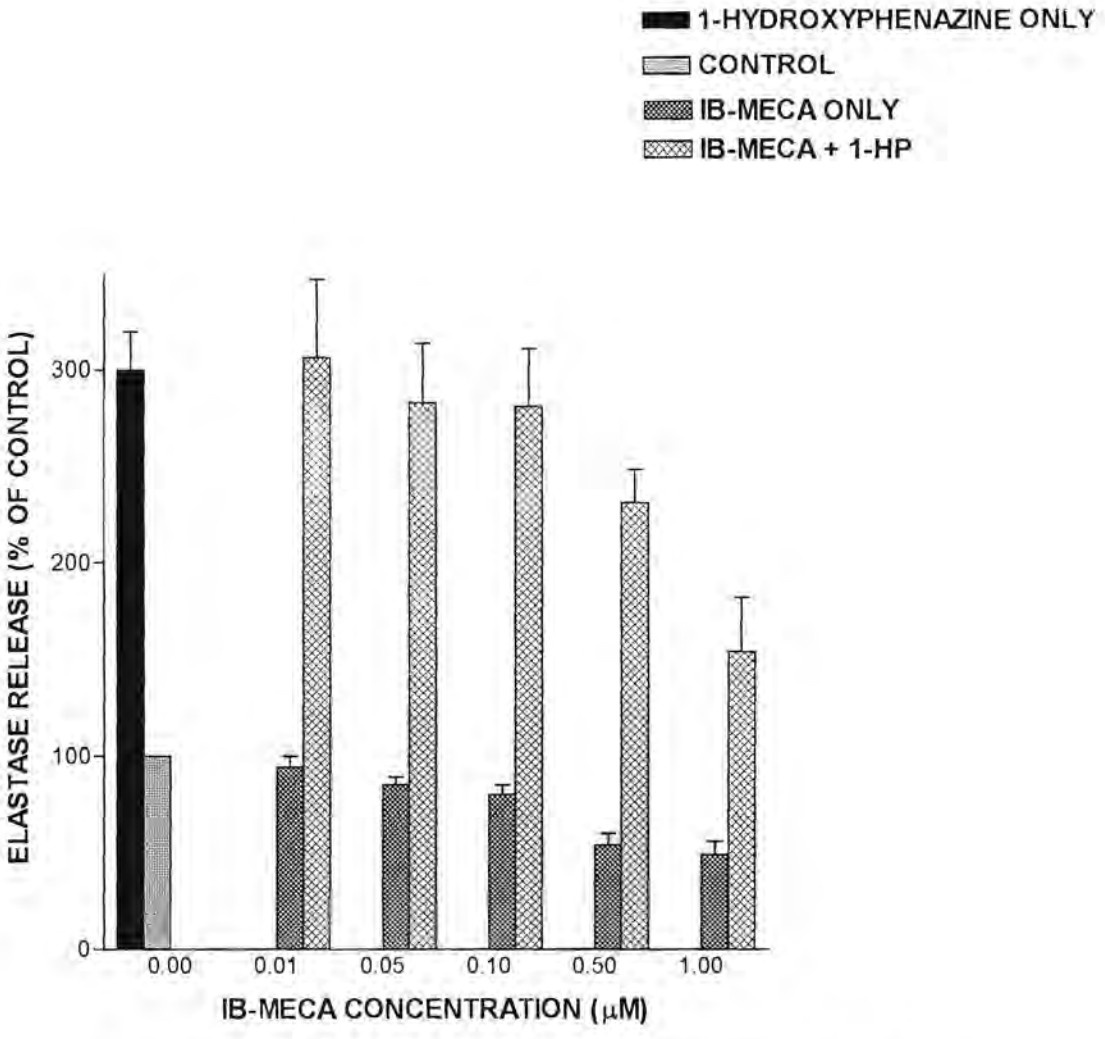


FIGURE 24: Effects of the A3 adenosine receptor agonist, IB-MECA (0.01-1μM) on the release of elastase from FMLP/CB-activated neutrophils in the presence and absence of 1-HP (12.5μM). The results of 4 different experiments are expressed as the mean percentage ± SEMs of the corresponding drug- and pigment-free control systems.

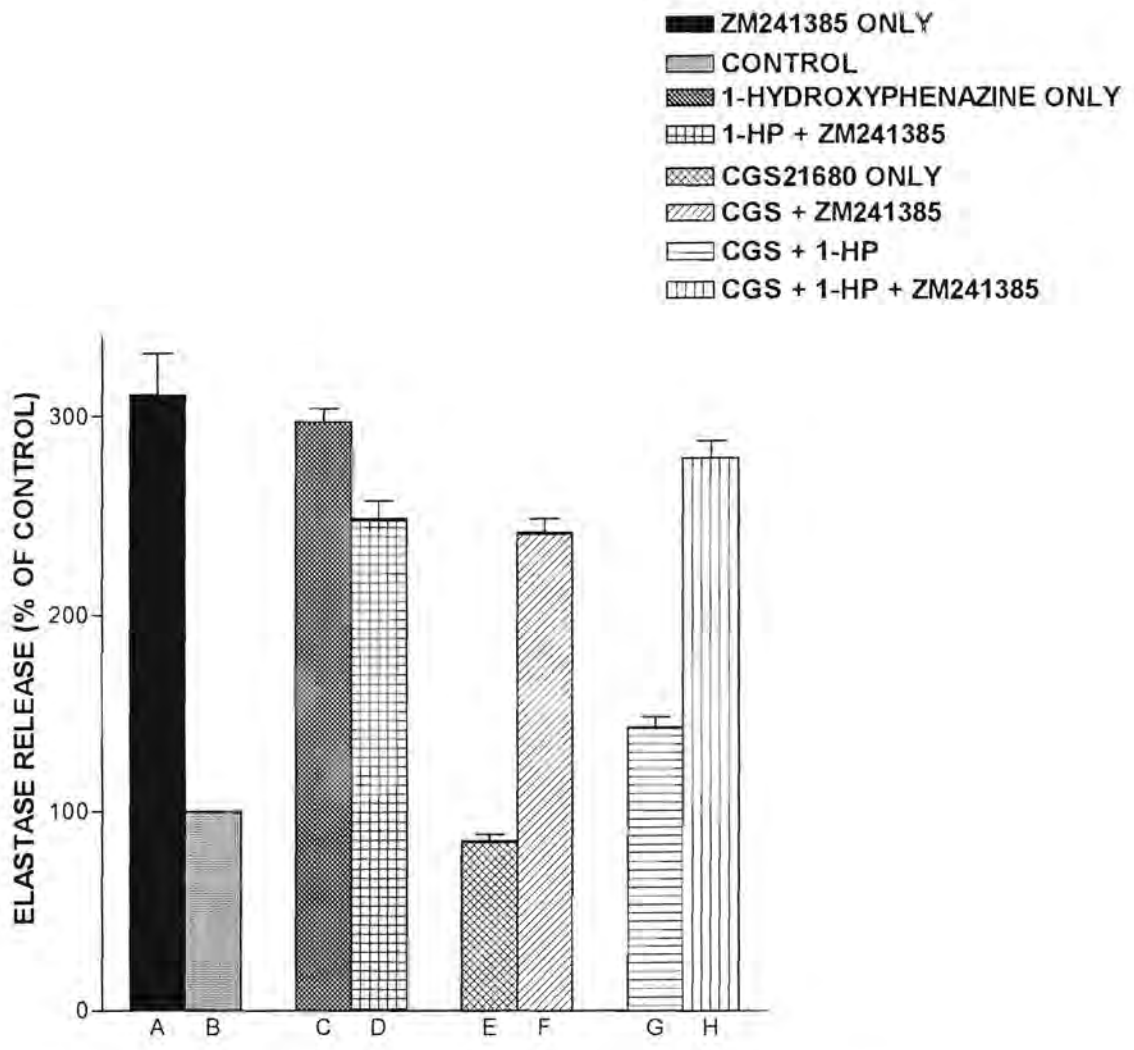


FIGURE 25: Effects of ZM241385 (2.5μM) on the release of elastase from FMLP/CB-activated control neutrophils and from neutrophils coincubated with CGS21680 (1μM) in the presence and absence of 1-HP (12.5μM). The results of a single representative experiment with 6 replicates for each system are expressed as the mean percentage ± SEMs of the corresponding drug- and pigment-free control systems. A = ZM241385; B = Control; C = 1-hydroxyphenazine(1-HP); D = 1-HP + ZM241385; E = CGS21680; F = CGS21680 + ZM241385; G = CGS21680 + 1-HP; H = CGS21680 + 1-HP + ZM241385

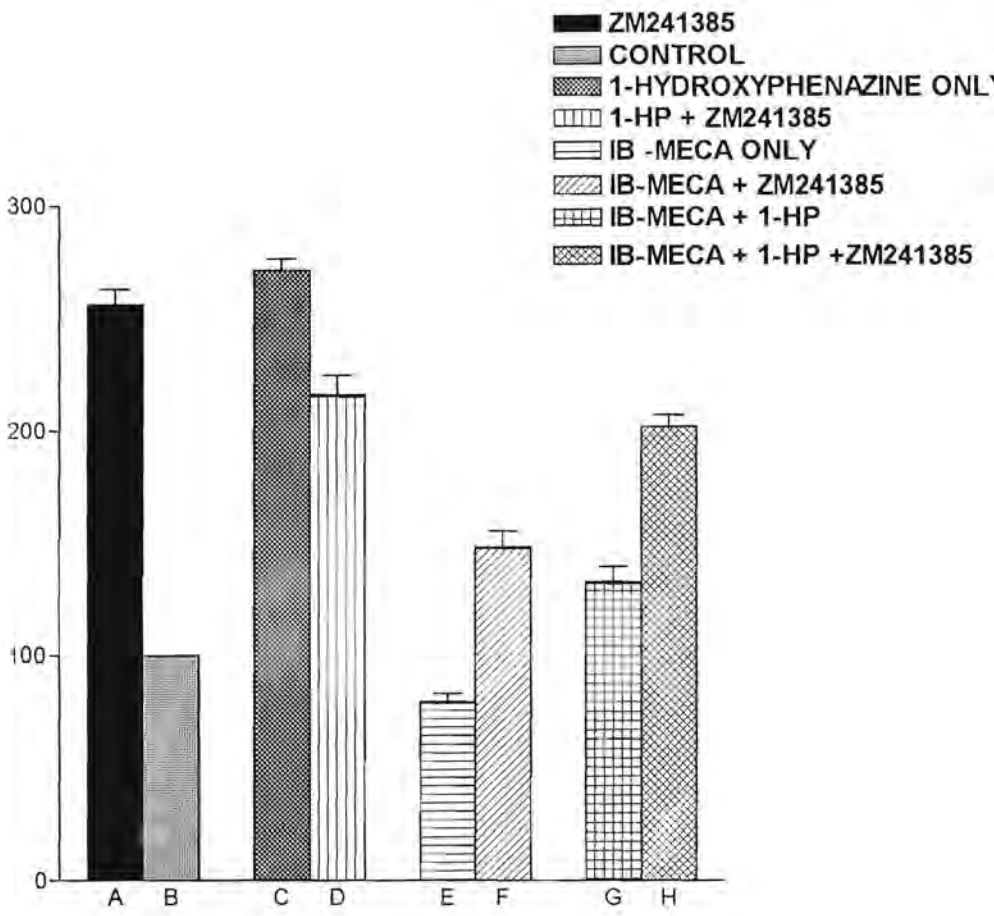


FIGURE 26: Effects of ZM241835 (2.5μM) on the release of elastase from FMLP/CB-activated control neutrophils and from neutrophils coincubated with IB-MECA (1μM) in the presence and absence of 1-HP (12.5μM). The results of a single representative experiment with 6 replicates for each system are expressed as the mean percentage ± SEMs of the corresponding drug- and pigment-free control systems. A = ZM241385; B = Control; C = 1-hydroxyphenazine (1-HP); D = 1-HP + ZM241385; E = IB-MECA (IB); F = IB-MECA + ZM241385; G = IB- MECA + 1-HP; H = IB-MECA + 1-HP + ZM241385.

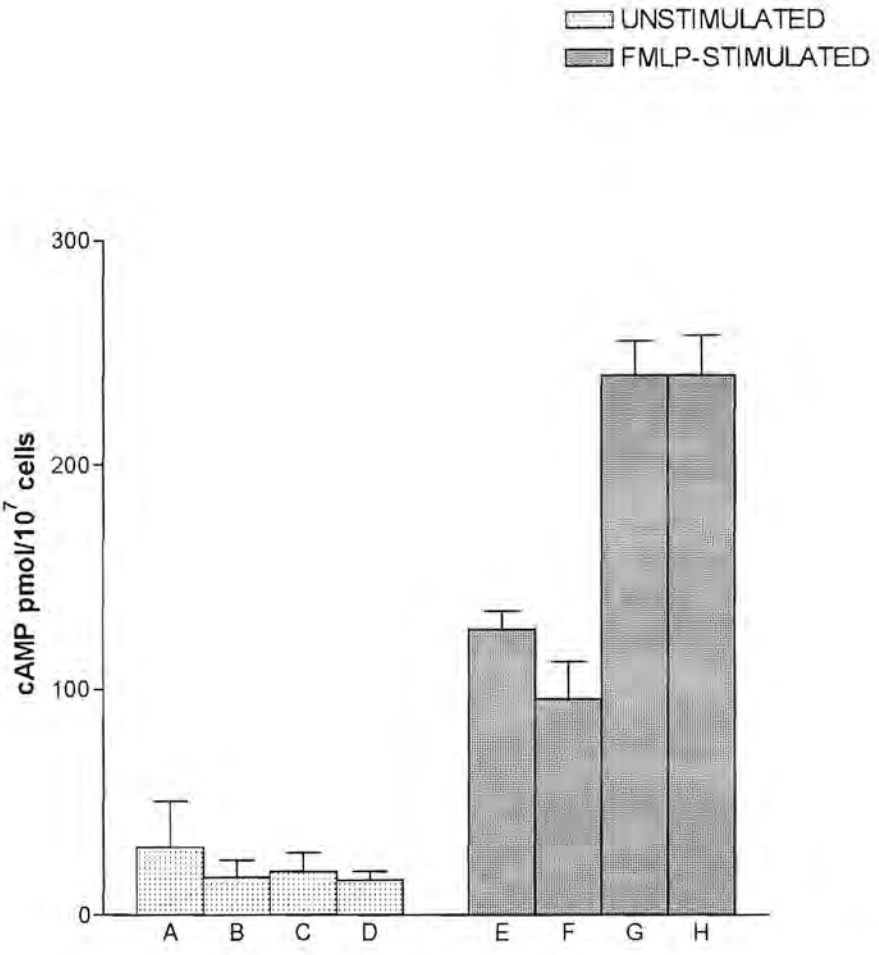


FIGURE 27: Effects of CGS21680 (1μM) on cAMP levels in unstimulated and FMLP-activated neutrophils in the presence and absence of 1-HP (12.5μM). The results of 4 different experiments are expressed as the mean cAMP concentration ± SEMs. A = Unstimulated neutrophils; B = Neutrophils + 1-HP only; C = Neutrophils + CGS21680 only; D = Neutrophils + CGS21680 + 1-HP; E -H = The corresponding systems for FMLP-treated neutrophils. The results shown are those for 1 min after the addition of FMLP.

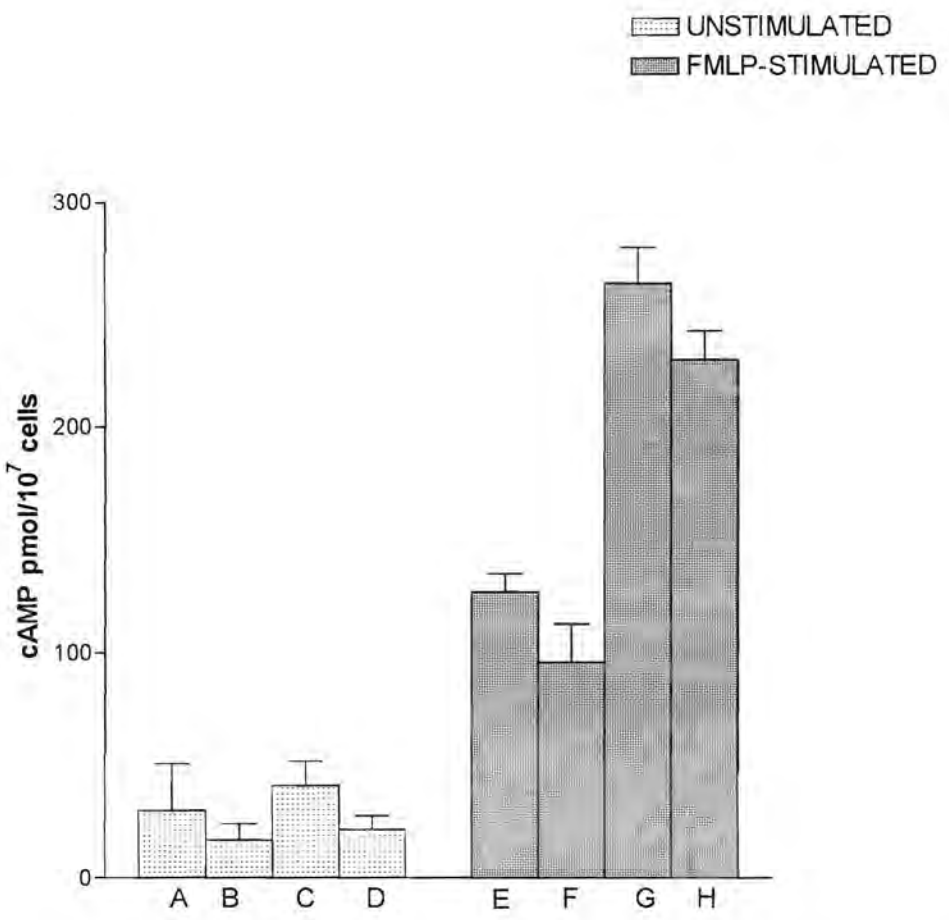


FIGURE 28: Effects of IB-MECA (1μM) on cAMP levels in unstimulated and FMLP-activated neutrophils in the presence and absence of 1-HP (12.5μM). The results of 4 different experiments are expressed as the mean cAMP concentration ± SEMs. A = Unstimulated neutrophils; B = Neutrophils + 1-HP only; C = Neutrophils + IB-MECA only; D = Neutrophils + IB-MECA + 1-HP; E-H = The corresponding systems for FMLP-treated neutrophils. The results shown are those for 1 min after the addition of FMLP.

4.4 DISCUSSION

The broad-spectrum anti-inflammatory potential of physiologic and pharmacologic cAMP-elevating agents, which spans many different types of immune and inflammatory cells, including neutrophils, has been recognised for more than two decades (Moore and Willoughby, 1995). However, the development of clinically useful cAMP-based, anti-inflammatory chemotherapeutic agents has, until recently, enjoyed limited success due to lack of selectivity for immune and inflammatory cells. Recent innovations include the second generation type 4 phosphodiesterase (PDE) inhibitors (Torphy, 1998; Underwood *et al.*, 1998) and adenosine receptor agonists operative at the level of type A2a receptors (Origini and Fredholm, 1996).

In *Pseudomonas aeruginosa* infections of the airways, the most dramatic change to occur is the rapid recruitment of neutrophils from the bloodstream. These phagocytes become the most numerous cell type in the airways of most chronically infected patients and are a source of a large burden of proteases, particularly elastase. As I have reported in the previous chapters, 1-HP, a proinflammatory phenazine pigment derived from *Pseudomonas aeruginosa,* potentiates the release of elastase from neutrophils. Since corticosteroids appear to have minimal, if any, counteracting effects on the pro-inflammatory activities of neutrophils (Cox, 1995; Wenzel, 1997), there is clearly a requirement for novel and effective chemotherapeutic agents which can target these cells. cAMP-elevating agents such as type 4 PDE inhibitors and adenosine receptor agonists show considerable promise in fulfilling this role.

In this chapter, the results of experiments designed to investigate the effects of A1, A2a, and A3 adenosine receptor agonists on the proinflammatory activity (elastase release) of 1-HP-treated and untreated neutrophils, the receptor specificity of these agents, as well as their effects on intracellular cAMP have been presented.

I have found that CPA, a selective A1 receptor agonist, showed no effect on elastase release (0.01-1μM) from neutrophils in either the presence or absence of FMLP, and neither did it affect cAMP levels. CGS21680 (selective A2a adenosine receptor

agonist) at 0.01-1μM and IB-MECA (selective A3 receptor agonist at 0.01-1μM) on the other hand, inhibited elastase release from both control and 1-HP-treated cells in a dose-dependent manner. Importantly, these anti-inflammatory effects of CGS21680 and IB-MECA were completely blocked by the highly selective A2a receptor antagonist, ZM241385, demonstrating the involvement of A2a receptors in the anti-inflammatory actions of both agonists (Hannon *et al.*, 1998; Sullivan *et al.*, 1995). This observation is in keeping with previous studies which have shown that IB-MECA interacts with both A2a and A3 receptors. Hannon *et al.*, (1998) demonstrated that ZM241835 at 0.01-10μM inhibited cAMP elevation in neutrophils as well as the inhibition of the oxidative burst induced by CGS21680 and 2CL-IB-MECA. It has also been shown by several groups (Cronstein *et al.*, 1985; Roberts *et al.*, 1985; Sullivan *et al.*, 1995; and Fredholm *et al.*, 1996) that one of the most marked effects on neutrophils activation of A2a adenosine receptors is inhibition of the generation of reactive oxidative species elicited by physiologic stimulants, including neutrophil chemoattractants, cytokines, and lipid products. Sullivan *et al.*, (1995) have also demonstrated that high concentrations (μM) of the A3 selective agonist, IB-MECA, produces anti-inflammatory responses that are mediated by A2a receptors.

Interestingly, ZM241385 *per se* potentiated the release of elastase from FMLP/CB-activated neutrophils. This observation supports the findings of a previous study in which it was reported that endogenously generated adenosine, acting via A2a receptors and cAMP, modulates the proinflammatory activities of activated neutrophils (Iannone *et al.*, 1989). Blocking of A2a receptors by ZM241385 could therefore be expected to antagonize the anti-inflammatory effects of endogenously generated adenosine, resulting in enhancement of degranulation as observed here.

My colleague, Dr Z. Visser has investigated the effects of the three adenosine receptor agonists on calcium fluxes in FMLP-activated neutrophils *in vitro*. It was found that none of the test agents affected the release of Ca^{2+} from intracellular stores in FMLP-activated neutrophils. However, CGS21680 and IB-MECA at the concentrations used here, but not CPA, accelerated the clearance of Ca^{2+} from the

cytosol of FMLP-activated neutrophils by causing accelerated resequestration of cytosolic Ca^{2+} by enhancement of the activity of the endomembrane Ca^{2+}-ATPase. These effects were antagonized by ZM241385 demonstrating the involvement of A2a receptors (Dr Z. Visser- personal communication).

I have also shown that both IB-MECA and CGS21680 but not CPA, increased cAMP levels in resting (IB-MECA) and FMLP-activated (CGS21680 and IB-MECA) neutrophils in both the presence and absence of 1-HP. Dr Visser also showed that the cAMP-elevating properties of both CGS21680 and IB-MECA were eliminated by the A2a receptor antagonist, ZM241385 (personal communication).

In summary, enhancement of elastase release from neutrophils mediated by the pro-inflammatory phenazine pigment, 1-hydroxyphenazine (1-HP), was neutralized and normalized by CGS21680 and IB-MECA. These effects are mediated by the interaction of these agents with A2a receptors and appear to involve cAMP. Selective activation of A2a adenosine receptors with novel pharmacologic agonists may be a useful strategy to ameliorate inappropriate inflammatory responses in chronic inflammatory disorders. CGS21680 does not fall into this category because it is not specific for A2a receptors (Hannon *et al.*, 1998). However, novel A2a receptor agonists with improved specificity have been designed and are currently undergoing phase I clinical trial (Dr M. Johnson, personal communication).

CHAPTER 5

CONCLUSIONS

5.1 CONCLUSIONS

In the current study, I have demonstrated that treatment of neutrophils with the pro-inflammatory phenazine pigment, 1-hydroxyphenazine (1-HP) at pathologically relevant concentrations of 0.38-12.5μM (Miller *et al.*, 1986; Ras *et al.*, 1992), potentiates the release of elastase and MPO from activated human neutrophils. Data obtained with the fura-2 fluorescence and radiometric procedures for measuring Ca^{2+} fluxes demonstrated that 1-HP compromised the efficiency of mechanisms involved in the clearnace of Ca^{2+} from the cytosol of activated neutrophils. This effect of 1-HP is associated with inhibition of efflux of the cation, due possibly to the antagonistic effect of 1-HP on the plasma membrane Ca^{2+}-ATPase.

Phospodiesterase type 4 inhibitors such as rolipram and adenosine receptor agonists operative at A2a receptors, for example, CGS21680, were able to neutralize 1-HP-mediated enhancement on elastase release and this was associated in the case of the former, with normalization of Ca^{2+} metabolism in 1-HP-treated neutrophils. This effect was probably achieved indirectly by increasing the activity of the endomembrane Ca^{2+}-ATPase which apparantely compensated for the decreased activity of the plasma membrane Ca^{2+} extrusion membrane system.

In conclusion, these cAMP-elevating agents cannot be used for the anti-inflammatory chemotherapy of patients colonized with *Pseudomonas aeruginosa*. However, second generation type 4 phosphodiesterase inhibitors and A2a receptor agonists are currently undergoing phase III (PDE 4 inhibitors) and phase I (A2a receptor agonists) assessment and may soon become available for clinical application. They may prove to be particularly useful in the treatment of chronic inflammatory disorders of both infective and non-infective origin (cystic fibrosis, bronchiectasis, chronic obstructive pulmonary disease, certain categories of asthmatics) which are poorly responsive to corticosteroids. They may prove to be particularly useful in neutralizing the proinflammatory actions of 1-HP in patients colonized with *Pseudomas aeruginosa,* a particularly intransigent microbial pathogen.