ALVEOLAR MACROPHAGES IN ASTHMA AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE

modulation of cellular activity

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ALVEOLAR MACROPHAGES IN ASTHMA AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE

modulation of cellular activity

ALVEOLAIRE MACROFAGEN IN ASTMA EN CHRONISCH ASPECIFIEKE RESPIRATOIRE AANDOENINGEN

modulatie van cellulaire activiteit

PROEFSCHRIFT

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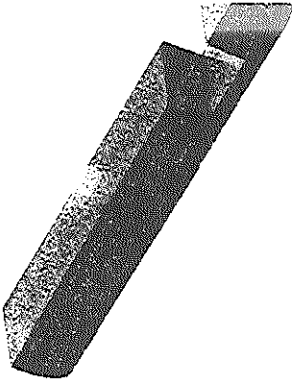
preface

Asthma and chronic obstructive pulmonary disease (COPD) affect over 10 % of the population in industrialized countries and its prevelance and mortality is still rising in stead of decreasing despite intensive drug therapy. This may be caused by the fact that both diseases are more than just bronchoconstriction but are the clinical manifestation of (a) very complex pathophysiological process(es). Indeed, over the past few years, investigators of several disciplins have focussed their attention to the underlying mechanisms that lead to the typical characteristics of both pulmonary diseases. One of the most striking similarities between asthma and COPD is their association with pulmonary inflammation which is regarded a fundamental event in the pathophysiology of asthma and COPD. This is reflected in the treatment of both diseases which, over the past few years, is clearly shifted from relief of symptoms (bronchodilators) towards a more causal therapy (anti-inflammatory drugs). Still, due to the complexity of mechanisms involved in pulmonary inflammation, asthma and COPD remain difficult to treat.

Pulmonary inflammation can be regarded as a complex puzzle consisting of an, as yet, unknown number of different pieces. Curing asthma and COPD or at least an adequate treatment of the disease implicates the full or partial elucidation of the puzzle. Since we do not know the full size or shape of the puzzle, one way to accomplish this, is gathering knowledge about the individual pieces which make up the unknown puzzle and trying to figure out how they fit together. In the pathophysiology of pulmonary inflammation the greater part of pieces consists of pulmonary cells and the interactions between them. Communication between cells is provided by means of interactions of a variety of mediators they produce which is attained through binding to cell-surface receptors. These receptors are part of ingenious mechanisms (transmembrane signalling systems) which translate external information into intracellular signals (second messengers). In turn, alterations in the concentration of second messengers modulate in a complex way the activity of the cell.

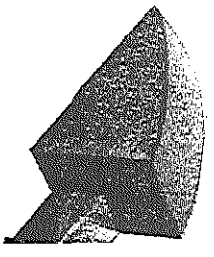
Having simplified a small part of the complexity of pulmonary inflammation to cells, mediators and second messengers, we have confined our study to the modulation of cellular activity of alveolar macrophages (AM), cells which exhibit an important key function in the processes of pulmonary inflammation. In the *second part* of this thesis (the first part contains a general introduction), the mechanisms by which inflammatory mediators and ß-adrenergic agonists interact with AM adenylyl cyclase (the transmembrane signalling system which produces the second messenger cyclic

AMP) and its modulation by immunologic challenge (sensitization and antigen challenge) are described. In the *third part*, the interactions of the lipid mediator platelet activating factor and AM are considered in more detail with a special reference to cAMP-production and arachidonic acid metabolism. In *part four*, the knowledge gathered from the previous parts has been employed to study the modulation of functional activity of human AM in which differences between AM from control subjects and COPD patients and asthmatics are emphasized. The thesis ends with *part five* which includes a general discussion and a summary.



PART ONE

GENERAL INTRODUCTION



chapter one
inflammation in asthma and chronic obstructive pulmonary disease

1.1. definitions and clinical aspects

Various classifications and definitions for asthma and chronic obstructive pulmonary diseases (COPD) have been proposed. Overlap in pathogenesis, pathophysiology, occurrence and clinical symptoms hampers to define asthma and COPD and the differences between them. Different symposia have been dedicated to reach a worldwide accepted classification of these pulmonary disorders [6,48,a].

1.1.1. asthma

Clinically, asthma can be characterized by increased responsiveness of the tracheobronchial tree to a variety of stimuli [6]. The major symptoms of asthma are expressed as attacks of dyspnea, wheezing and cough, which may vary from mild and almost undetectable to severe and unremitting symptoms (status asthmaticus). Asthmatic subjects develop a greater degree of bronchoconstriction after exposure to various stimuli than do subjects with normal bronchial reactivity. This excessive reactivity of the airways to a large variety of physical, psychological, chemical, and pharmacological stimuli is generally refered to as bronchial hyperreactivity (BHR). The primary physiological manifestation of BHR is variable airway obstruction. The severity of symptoms of asthma appears to correlate closely with the degree of hyperreactivity which emphazises the fundamental role of BHR in this disease [142]. Finally, these stimuli are often used to classify asthmatics into atopic and non-atopic subjects. Stimuli like methacholine or histamine provoke BHR in any subject with asthma while more specific (immunological) stimuli, like house dust or grass pollen affect only atopic asthmatics. Generally, atopy refers to the inherited tendency to produce specific antibodies (of the IgE class) to such common environmental antigens [197,207].

1.1.2. chronic obstructive pulmonary disease

Chronic Obstructive Pulmonary Disease or COPD is the collective designation for three pulmonary disorders: emphysema, peripheral airway disease, and chronic bronchitis. Clinically, these diseases are characterized by constant abnormal

expiratory flow (obstruction) over several months and is frequently associated with cough, wheezing, increased sputum production, and recurrent respiratory infections. Long-term cigarette smoking is the principal identified risk factor for COPD [212]. However, these disorders do not exclusively occur in cigarette smokers as the majority of them do not develop any -clicallly manifested- lung disease [37]. Occupational and social environment may form additional risk factors to develop COPD. Furthermore, the inherited deficiency of plasma α_1- antiprotease renders the patient more susceptible to the damaging effect of cigarette smoke and induces the early development of COPD [40,136,244].

emphysema

Emphysema is defined as a condition of the lung characterized by abnormal permanent enlargement of the alveolar airspaces distal to the terminal bronchiole. It is accompanied by destruction of lungtissue whereas obvious fibrosis is absent [6]. Emphysema can be subclassified in three subtypes: 1) centricinar or proximal acinar emhysema in which the proximal part of the acinus is predominantly involved; 2) panacinar emphysema in which all components of the acinus tend to be involved equally. It is the form of emphysema commonly associated with α_1- antiprotease deficiency [40] and 3) distal acinar emphysema in which the distal part of the acinus, alveolar ducts and sacs, are predominantly involved.

peripheral airway disease

Peripheral airway disease is ill-defined as it is not a disease per se. The term is commonly used to characterize the morphological abnormalities in the peripheral airways which precede the clinical manifestation of emphysema or chronic bronchitis such as inflammation of the terminal and respiratory bronchioles, fibrosis of airway walls, and goblet cell metaplasia of the bronchiolar epithelium [243].
In cigarette smokers, pathological changes in the peripheral airways appear to precede the development of emphysema [198]. Small local inflammation of the airway walls may lead to physical and functional impairment of the airflow and may as such be manifested as "early or preclinical" COPD [57,271].

chronic bronchitis

Chronic bronchitis refers to a condition of subjects with chronic or recurrent excess mucus secretion into the bronchial tree [6]. Excess mucus secretion was empirically recognized as the production of any sputum, whether expectorated or swallowed, and frequently sputum production is accompanied by chronic cough.

Asthma and COPD apparently show many similar clinical characteristics which renders the distinction between these pulmonary diseases difficult and arbitrary. This distinction becomes even more difficult when pathological and histological examinations of airways from asthmatics and COPD patients are included. In both diseases, the airways show increased mucus secretion [72,190], the formation of edema as a result from plasma exudation [121,190,210], thickening of the basement membrane [137], smooth muscle hypertrophy [78,131], and infiltrates of inflammatory cells [20,101], all typical features of pulmonary inflammation.

1.2. inflammation in asthma and copd

Inflammation is the response of vascularized tissue to injury which serves to resolve and repair the effects of damage. Factors, which may initiate pulmonary inflammation are diverse and include infectious agents (bacteria, viruses), physical stimuli, chemical agents (drugs, toxins, industrial pollutants) and allergens. The histopathological features of inflammation involve changes in vascular blood flow, alterations in vascular permeability leading to plasma exudation and the formation of edema. In addition, a cascade of events occur which involve a variety of inflammatory cells such as macrophages and neutrophils. Thus, after leaving the blood compartiment, inflammatory cells migrate under the influence of chemotactic agents to the site of injury and induce the process of repairment. The major link between the occurrence of injury and the onset of inflammation consists of mediators derived from plasma, injured tissue or inflammatory cells. Thus, kinins, complement components, prostaglandins and leukotrienes serve as messengers to recrute and activate different cell types. With respect to duration and nature, inflammation is generally classified as acute and chronic inflammation. The acute response is characterized principally by the vascular and exudative changes as described above whereas chronic inflammation results from more persistent injurious stimuli (often weeks or months)

leading to a predominantly proliferative rather than an exudative reaction. Chronic inflammation may follow acute inflammation (when the acute inflammatory response cannot be resolved) or may be chronic almost from the onset leading to extensive tissue destruction and fibrosis.

Originally, evidence for the implication of inflammatory processes in the pathogenesis of asthma and COPD has been derived from studies using tissues from patients who had died in status asthmaticus [72,103]. The advanced technique of broncho alveolar lavage (BAL) and bronchial biopsy recently enables to study the involvement of inflammatory processes in more moderate forms of both asthma and COPD.
The cellular events in pulmonary inflammation are reviewed in the next paragraphs (alveolar macrophages will be discussed in more detail in chapter two).

Differences in the number and differentiation as well as the activity of the cells and the presence of inflammatory mediators are observed when BAL fluids of asthmatics and COPD patients are compared to normal subjects. The number of eosinophils and the concentrations of eosinophil major basic protein (MBP) were increased in BAL fluids of asthmatics as compared to normal subjects [245,261]. In addition, elevated numbers of eosinophils and neutrophils were observed in BAL- fluids of atopic asthmatic subjects who developed a late phase fall in FEV_1 following allergen bronchial challenge [67,69,80,186]. Activation of peripheral blood neutrophils was also found in on-going asthma and after allergen bronchial challenge and exercise-induced asthma [74,102,187]. Mast cell numbers were increased in the BAL- fluids of asthmatics as compared to normal controls [245,261] and the number could be correlated with the degree of BHR [261]. In addition, mast cells from asthmatics show elevated releasability as compared to those from normal subjects [245]. Alveolar macrophages isolated from asthmatics show enhanced expression of low affinity IgE receptors, enhanced lipid mediator release and enhanced exocytosis of granule-derived proteases in response to specific allergen and anti-IgE challenge as compared to normals [96,104,141].
In COPD, most observations relating to inflammatory cells concerned neutrophils and macrophages. Increased numbers of neutrophils were observed in BAL fluids of asymptomatic smokers and those with chronic cough and sputum production [180]. The amounts of superoxide ion generated in response to phorbol myristate acetate (PMA) stimulus by peripheral blood neutrophils were shown to correlate with the degree of

non-specific BHR in COPD patients [214]. Putative neutrophil membrane-derived mediators such as LTB_4 were shown to be present in sputum of COPD patients [273]. BAL macrophages from smokers and COPD patients showed significantly elevated elastase release as compared to normal control [130,220]. Cathepsin B-like cysteine proteinase activity was described in COPD- sputum and localized histochemically to alveolar macrophages [185].

Despite the many indications for a role of inflammatory cells in asthma and COPD, it remains to be estabilshed to what extent inflammatory cells and their products are involved in BHR and asthma and how, in COPD, inflammatory cells can cause small airways obstruction and emphysema. Thus, the relationship between inflammatory infiltration, cellular activation and disease severity in asthma and COPD is still not clear. To assess the contribution of these cells to the pathogenesis of asthma and COPD, more data are needed on the functional properties (e.g. cellular activity and mediator releasability in response to specific and non-specific stimuli) of different cells directly related to the site and nature of airway inflammation.

1.2.1. cells in pulmonary inflammation

Several types of inflammatory cell have been implicated in the pathogenesis of asthma and COPD. What remains unclear is how the different cellular components interact with each other to induce the pathological symptoms of asthma and COPD (figure 1.1.). Obviously, after specific or non-specific stimuli, inflammatory mediators are generated from 1) cells normally found in the lung, such as alveolar macrophages, mast cells, and epithelial cells (primary effector cells), and 2) from cells recruited into the lung, such as eosinophils, neutrophils, lymphocytes, platelets and interstitial macrophages (secondary effector cells).

The putative contribution of the different inflammatory cell types and their mediators to the pathogenesis of inflammatory processes in asthma and COPD is discussed below.

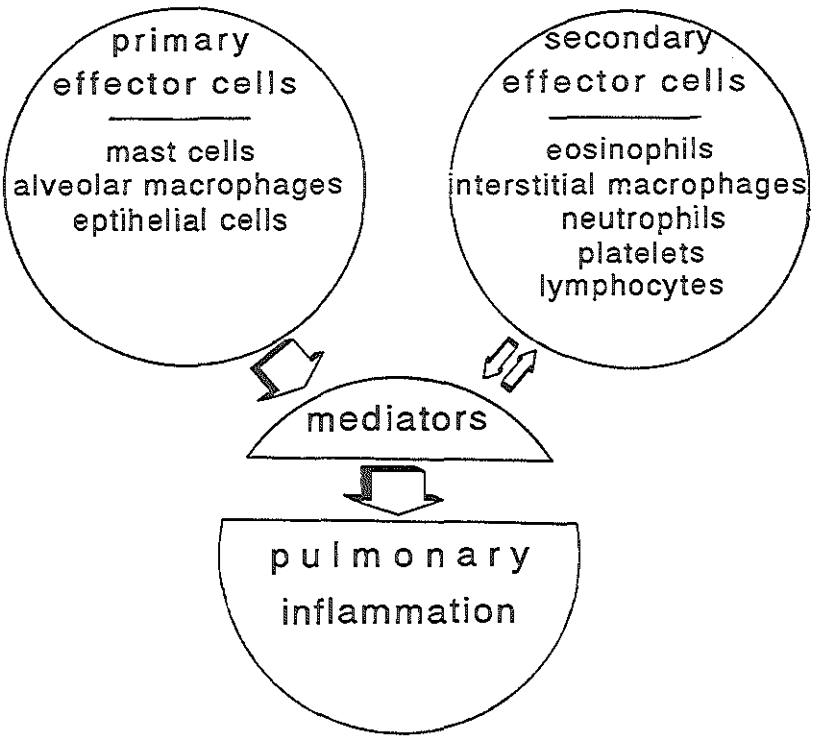


Figure 1.1. Subdivision of pulmonary inflammatory cells into primary effector cells and secondary effector cells.

primary effector cells

mast cells

Mast cells are polymorphnuclear leucocytes that differentiate in the bone marrow and are so called 'end cells' (cells incapable of division) with a lifespan of approximately 10 days. Mast cells are found throughout the respiratory tract, most of them located associated with the walls of the alveoli (40%) and airways (60%) [143]. The majority of mast cells in the airways are located between the bronchial epithelial cells and the

basement membrane [156] and occassionally between epithelial cells and on the surface of the airways [138].
Mast cells in the bronchial epithelium and submucosa of normal airways contain secretory granules in which tryptase is the predominant neutral protease. These cells have been designated MC_T to differentiate them from those containing both tryptase and chymase (MC_{TC}). More than 95 % of the epithelial mast cells and 75 % of the subepithelial mast cells in human airways are of the MC_T- subtype [231,232].

Perturbation of the high affinity IgE-Fc receptors ($Fc_\epsilon R_1$) by allergen or other stimuli induces mast cells to secrete mediators which are involved in pulmonary inflammatory processes. These mast cell-derived mediators can be divided into two mean catagories: pre-formed or granule-associated mediators and the newly-formed or membrane-derived mediators [165]. Pre-formed mediators include histamine, various lytic enzymes and chemotactic peptides such as neutrophil chemotactic factor of anaphylaxis (NCF-A) and eosinophil chemotactic factor (ECF). The membrane-derived mediators are mainly metabolites of arachidonic acid and platelet activating factor (PAF). The most important cyclo-oxygenase product of human mast cells is prostaglandin D_2 [38,124,230] though other cyclo-oxygenase metabolites have been reported [77,230]. LTB_4, and the cysteinyl leukotrienes C_4, D_4 and E_4 are the mean lipoxygenase metabolites released from human mast cells. Pulmonary mast cells are highly heterogeneous in both functional and structural respect. Consequently, the amount of mediators released can vary between mast cells of the bronchi, the alveoli and the pulmonary parenchyma [70,126]. Thus, mast cells recovered from BAL have a much reduced capacity to generate LTC_4 when these are compared to mast cells dispersed from human lung parenchyma [2,91].
In the lungs of patients with allergic asthma, a three- to fivefold increase in the relative numbers of pulmonary mast cells have been reported [87,245,261] whereas histological examination of the airways from patients who died during a severe attack of asthma demonstrated increased numbers of degranulated mast cells [55].

Although mast cells produce a variety of mediators, more recent evidence argues against an important role for these cells in the pathogenesis of asthma (and COPD). Cromolyn sodium and the β_2- adrenoceptor agonists inhibit mast cell degranulation [46] and block the early response but fail to inhibit the late responses (not cromolyn sodium) or to prevent or reduce BHR [52,148,150]. In addition, corticosteroids which are

highly effective in preventing the late response and reduce BHR, have no direct action on human lung mast cells and do not block the early response [76,227].

Thus, although mast cells are clearly involved in the immediate responses to allergen (and possibly other acute responses like exercise), they seem to be less crucial for the maintenance of pulmonary inflammatory processes.

alveolar macrophages

Alveolar macrophages (AM), derived from blood monocytes are one of the family of mononuclear phagocytic cells which are found in virtually every tissue. There are a number of reasons to suggest AM as a primary effector cell in asthma and COPD. First, airways from normal subjects, asthmatics and COPD- patients are lined with AM [11]. It has been estimated that up to 500 million cells may be present in the human airway from the larynx to the alveoli. Although the proportion of cell types may differ between normals, asthmatics and COPD patients, AM make up over 90 % of the cell population of the airways [11]. They would therefore be the most predominant inflammatory cells to be exposed to inhaled antigens.
Secondly, AM possess low affinity IgE receptors [184] and upon *in vitro* IgE challenge human AM can be activated and will release mediators [96,141]. This activation of AM via IgE- receptors induces the release of a wide range of inflammatory mediators, including spasmogens (like thromboxane A_2 , prostaglandin D_2 and prostaglandin $F_{2\alpha}$), enzymes, chemotactic agents (p.e. leukotriene B_4, platelet activating factor, complement C3a), and cytokines [7,10,96,177].
Lastly, AM may participate in the immune response either by acting as antigen presenting cells or as response cells. AM present many different surface receptors for immunoglobulins and cytokines, which upon binding modulate the cell to act either suppressive or as an amplifier in the immune response.

In chapter 2, the origin, typical features and functions of AM in the pulmonary inflammatory processes will be discussed in further detail.

epithelium

Until recently, epithelial cells were considered to be inert as the physical layer covering the airways. It is now apparent that these cells with their key location on the interface between the external environment and the internal milieu have an important function in the defence of the airways and pulmonary inflammatory processes. Associated with inflammatory processes, epithelial damage is considered to be an important feature in asthma [154]. Loss of epithelium increases the actions of spasminogens *in vitro*, possibly because airway epithelial cells release relaxing factors [15,27,86,61,122,193,254] or by exposing sensory nerve endings, which lead to local and cholinergic reflex bronchoconstriction [16]. In addition, epithelial cells are capable of generating inflammatory mediators like LTB_4 and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) which are chemotactic for inflammatory cells [127,132,149,237] and produce PGE_2 [153] and are likely to produce PAF in an analogeous way to endothelial vessel cells [183].
Besides, airway epithelial cells have multiple effects on airway smooth muscle. Intact epithelium inhibits muscle tone by releasing PGE_2. Human and canine airway epithelial cells in vitro produce low concentrations of PGE_2, but when they are stimulated by inflammatory mediators, like bradykinin [153] or by eosinophil MBP [135] these cells produce large amounts of PGE_2 which have profound effects on smooth muscle [18].

In asthma, epithelium disruption may contribute to BHR in a number of ways [61,140]. Thus, epithelial cell stimulation under conditions associated with damage by other mediators could produce lipoxygenase products which in turn stimulate other inflammatory cells. In addition, loss of epithelium may lead to increased permeability to antigens [122], exposure of sensory nerve fibers and actuation of local reflex mechanisms [16], changes in osmolarity of the bronchial surface lining fluid [122] and decreased production of putative epithelial-derived relaxant factors [16,86].

Although the putative role of epithelial cells is only beginning to become apparent and needs further elucidation, it is clear that bronchial epithelium is more than a protective barrier and that its capacity to generate inflammatory mediators enables it to play an active role in bronchial inflammatory processes.

secondary effector cells

eosinophils

Eosinophils are bone marrow-derived 'end cells'. They are present in large numbers in the circulation and tissues in those conditions associated with raised plasma IgE-levels, such as hay fever, allergic asthma, allergic bronchopulmonary aspergilloses and helminth parasitic disease [95,147]. The cytoplasmatic granules of eosinophils are rich in peroxidase and other basic proteins, i.e. major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO). MBP and ECP are cytotoxic towards bronchial epithelium [93,94] and have been shown to be elevated in sputum and BAL fluids of asthmatics [94,261]. They have therefore been implicated in the pathogenesis of epithelial disruption in asthma [155]. EPO may prime AM for more effective killing of microorganisms [215].

Eosinophils have the capacity to generate 5- and 15- lipoxygenase products [158,245] and the potent inflammatory mediator PAF [182]. These agents, particularly PAF, are powerful chemotactic factors for the eosinophil itself. In contrast to human neutrophils, which release LTB_4 as their major 5-lipoxygenase product, human eosinophils preferentially generate and secrete LTC_4 and only a small amount of LTB_4 [264]. Priming of eosinophils via prior exposure to chemotactic stimuli like LTB_4, PAF, interleukin 3 (IL-3), IL-5 or granulocyte-macrophage colony stimulating factor (GM-CSF) enhances the ability to release LTC_4 [264].

Particularly, eosinophils are implicated in the late phase asthmatic reactions [260] as their number recovered by BAL was increased in asthmatics [67,186,261] and the level of its secretory products was increased as well [261].

neutrophils

Neutrophils are non-dividing, granule containing cells which arise in the bone marrow and have a limited lifespan (6-8 hr) in the circulation. Besides its phagocytic capability, the neutrophil is a secretory cell. The neutrophil granules contain a variety of lytic enzymes, $O_2^{\cdot}$-radicals and related products which may contribute to the disruption and shedding of the airway epithelium [233,262,263]. The neutrophil is also

capable of synthezising a number of membrane derived mediators. The major metabolite of arachidonic acid formed by this cell is LTB_4 whereas LTC_4, 5-HETE, PGE_2 and TxA_2 are also produced by this cell [71,225,257]. Furthermore, these cells are capable of producing and secreting PAF [176].

Neutrophils are very rapidly mobilized into the lung in response to inflammatory reactions as the vascular bed of the lung contains a particularly large leukocyte pool. The MAC_1/CR_3 surface protein appears to be the component on the neutrophil surface primarily responsible for binding to the microvasculature [123]. Chemotactic agents rapidly increase the availability of this protein for binding thereby promoting the interaction of neutrophils with endothelial cells of the vessel wall in the capillary and postcapillary regions. This process can be stimulated by bacterial lipopolysaccharides and cytokines, like IL-1, TNF and γ-interferon [114].

The role of neutrophils in the pathogenesis of both asthma and COPD is still unclear but due to their ability to produce a variety of inflammatory mediators and related products, it seems conceivable to implicate these cells in both diseases. In serum of asthmatic patients, neutrophil chemotactic activity (NCA) has been observed during bronchospasm induced by exercise or antigen inhalation [162,194]. In addition, activated neutrophils have been shown to induce epithelial cell damage [239]. In COPD, neutrophils have been associated with the development of pulmonary emphysema. One hypothesis, originally described by Laurell and Eriksson [157], states that destruction of lung parenchyma in emphysema may be due to the local digestion of lung tissue by the action of proteinases. Neutrophils are a rich source of proteinases that can digest elastin [51,160]. Besides damaging lung tissue, oxidants generated by neutrophils can inactivate antiproteinase [41], which was found to be decreased in lavage fluids from smoking emphysema patients [42].

platelets

Platelets, derived from megakaryocytes, are non-nucleated cells containing a large number of granules which play an essential role in blood clotting. The granules contain a variety of substances which may act as mediators of inflammation such as proteolytic enzymes, cationic substances, ADP and serotonine which causes smooth muscle contraction and increases vascular permeability [39]. In addition, platelets have

the ability to generate membrane-derived mediators like PAF [24] and arachidonic acid metabolites such as prostaglandins, thromboxanes and 5-lipoxygenase products [192,248]. The release of active substances from platelets is a complex process which may be initiated by adherence and aggregation. Platelets can be activated by collagen, thrombin, ADP as well as PAF, derived from other inflammatory cells. Platelets possess IgG receptors [119] and low affinity IgE- receptors, suggesting that these cells might be specifically sensitized in allergic diseases in a comparable fashion to mast cells, eosinophils and macrophages [188].

The mechanisms by which platelets may affect airway function remain to be elucidated; the close apposition of these cells to airway smooth muscle in guinea pigs challenged with PAF [163,255] suggests that they may have a direct effect through the release of mediators.

lymphocytes

Lymphocytes can be divided into two catagories: thymus- derived lymphocytes, or T-cells, which are concerned with cell-mediated immunity (e.g. delayed hypersensitivity), and bone marrow-derived lymphocytes or B-cells. B-cells mature into antibody-forming plasma cells which can secrete all immunoglobulin subclasses, including IgE. B-cells complete their differentiation within the bone marrow, with final maturation taking place in the peripheral lymphoid organs such as the spleen, lymph nodes and gut associated lymphoid tissues (GALT). T-cell maturation involves T-cell progenitors which also arise in the bone marrow but further differentiate and proliferate in the thymus to give mature T-cells.

Upon presentation of antigen and surface contact, B-cells proliferate and differentiate to plasma cells. Differentiated B-cells participate in the pulmonary immunological defence through the production and secretion of proteins and a variety of antibodies, including immunoglobulins of the IgE-class.

T-cells are initially primed by the recognition of an antigen and "self", presented by macrophages. Thus, macrophages first process antigen (recognize and phagocytose) and then present it, on their cell surface, in combination with HLA- antigens ("self") to T-cells. This interaction leads to T-cell priming, differentiation and proliferation with

the formation of T-lymphoblasts and the secretion of soluble mediators called lymphokines.

It is becoming more and more apparent that pulmonary lymphocyte-associated mechanisms are involved in asthma and COPD. Still, these cells have received suprisingly little attention and only few studies have suggested a putative role of T-cells in allergic asthma. These studies have reported changes in the T-cell subsets in BAL fluids and peripheral blood from asthmatics [100,109,186]. Furthermore, activity of CD4+ T-cells is increased in asthmatics as shown by enhanced production of interferon-γ (IFN-γ) [92], interleukin-4 (IL-4) and IL-5, three interleukins known to be involved in the regulation of IgE production [170]. In addition, T-cell derived lymphokines promote the production of eosinophils by the bone-marrow (IL-3, IL-5 and GM-CSF), and modulate mast cell differentiation [120]. Interleukin 1 and platelet derived growth factor (PDGF) are chemotactic for inflammatory cells, like neutrophils, eosinophils and monocytes [259].

interstitial macrophages

Due to difficulties in harvesting pure populations of interstitial macrophages (IM), little is known about these cells. They are derived from blood monocytes and probably reside within the lung parenchyma, differentiating them from AM although some investigators consider IM as immediate precursors for the AM [1,28], especially during episodes of severe pulmonary inflammation [29,30,227]. Thus, whether IM can be regarded a macrophage population distinct from AM or as some kind of intermediate cell type between blood monocytes and AM remains to be elucidated. Though data are still lacking, the putative role of IM as effector cells in pulmonary inflammation may be deduced from functional characteristics of blood monocytes and AM (described in the chapter 2). Purlely based on their anatomical location (within lung parenchyma) IM may be classified as secondary effector cells.

1.2.2. mediators in pulmonary inflammation

The role of each mediator itself is complex and their mutual interactions are even more complex. Bearing this in mind, the most important features of the mediators relevant for asthma and COPD are reviewed in the following paragraphs. Emphasized

in this summary are synthesis, metabolism, binding to specific receptors, their action on pulmonary cells and their pathophysiological role in asthma and COPD.

Table 1.1. Mediators and some of their characteristics in pulmonary inflammation *.

agent		action
histamine		smooth muscle contraction vasoconstriction (H_1) vasodilatation (H_2) microvascular leakage chemotactic for inflammatory cells
prostaglandins	PGE_2	smooth muscle relaxation down-regulation of cellular activity vasodilatation
	PGE_1	smooth muscle relaxation vasodilatation inhibits platelet aggregation
	PGI_2	smooth muscle relaxation
	PGD_2	smooth muscle contraction
	$PGF_{2\alpha}$	smooth muscle contraction
	TxA_2	smooth muscle contraction vasoconstriction
leukotrienes	LTB_4	chemotactic for inflammatory cells increases microvascular permeability stimulated edema formation
	LTC_4	smooth muscle contraction stimulates mucus secretion increases microvascular permeability stimulates edema formation
	LTD_4	smooth muscle contraction stimulates mucus secretion increases microvascular permeability stimulates edema formation
	LTE_4	smooth muscle contraction stimulates mucus secretion increases microvascular permeability stimulates edema formation
	15HETE	smooth muscle contraction chemotactic for inflammatory cells
platelet activating factor		smooth muscle contraction increases microvascular permeability stimulates mucus secretion stimulates edema formation chemotactic for inflammatory cells
neuropeptides	VIP	smooth muscle relaxation decreases edema formation vasodilation
	SP	smooth muscle contraction stimulates mucus secretion increases microvascular permeability stimulates edema formation
complement	C3a	(indirect) smooth muscle contraction increases (indirect) vascular permeability increases (indirect) mucus secretion
	C5a	(indirect) smooth muscle contraction chemotactic for inflammatory cells increases (indirect) vascular permeability

* *abbreviations* are clarified in text.

Their interactions with receptors and subsequent involvement of transmembrane signalling systems will be discussed in chapter three.

Inflammatory mediators may have a large spectrum of effects on a variety of target cells in the airways, which are relevant in asthma and COPD. Some of them may directly lead to contraction of airway smooth muscle or enhance muscle tone and indirectly, via the secondary release of mediators or neuronal substances. They may also lead to increased secretion from submucosal glands, to increased fluid transport across airway epithelium, and to increased microvascular leakage. These events result in edema of the airways and exudation of plasma into the airway lumen, which itself may lead to the formation of new mediators [208]. Inflammatory mediators may attract and activate inflammatory cells which conversely may release a whole array of mediators serving to perpetuate and emphasize the inflammatory response (table 1.1.).

histamine

Histamine was implicated in the pathogenesis of asthma shortly after its discovery early this century when it was shown to mimic anaphylactic bronchoconstriction in guinea pigs [65]. Later it was shown to cause bronchoconstriction in asthmatics but not in normal subjects [60]. It is now well established that histamine is released in asthmatic patients upon bronchial challenge, and plasma levels of histamine are elevated in both atopic and non-atopic patients [12].
Histamine is formed by decarboxylation of the natural amino acid histidine and stored in preformed cytoplasmic granules of mast cells and basophils [218]. Histamine forms 5 to 10 % of the content of human mast cell granules and it can be released by a calcium dependent and active secretory process induced by both chemical and physical stimuli [204,268].

Histamine produces its effects by interacting with specific receptors on target cells. To date there are three subtypes of histamine receptors decribed, designated as H_1, H_2 and H_3-receptors. H_1-receptors have been identified in animal and human lung homogenates by receptor binding techniques [43,44,110]. In addition, H_1-receptors have been localized to airway epithelial cells and macrophages [236]. H_2-receptors have been identified in animal lung homogenates but their precize localization is not well

known [88]. H_3-receptors have been differentiated from H_1 and H_2-receptors using the selective agonist α-methyl histamine and the antagonist thioperamide, but the role of pulmonary H_3-receptors remain to be established.

Histamine contracts both large and small human airways *in vitro* [85].In animals this contractile effect seems to be modulated by airway epithelium [61,86]. Inhaled histamine causes bronchoconstriction in asthmatic patients more readily than normal subjects which is a manifestation of bronchial hyperreactivity [27]. On human pulmonary vessels, histamine has a dual effect with constriction mediated by H_1-receptors and vasodilatation via H_2-receptors [25]. Histamine also causes microvascular leakage in the bronchial microvasculature via activation of H_1-receptors [209,226]. The same receptor subtype is involved in the histamine-induced increase in action potentials in intrapulmonary vagal afferent nerves [224]. Histaminergic effects on other cells are confined to chemotactic effects on eosinophils [50,247] and neutrophils [234], the possible stimulation of T-lymphocyte suppressor cell function (via H_2-receptors) [21] and a self-limiting effect on the IgE-mediated release from human basophils [168].

arachidonic acid metabolites

Arachidonic acid derives from phospholipids in membranes and may be metabolized via two major enzymatic pathways (figure 1.2.). Arachidonic acid is oxidized by *cyclooxygenase* to the cyclic endoperoxide PGG_2, which is rapidly reduced to another unstable endoperoxide PGH_2, which then gives rise to $PGF_{2\alpha}$, PGE_2 and PGD_2. Another enzymatic pathway for cyclic endoperoxides leads to the formation of thromboxane A_2 (TxA_2) and prostacyclin (PGI_2), which are both unstable and are rapidly hydrolyzed to the inactive but stable TxB_2 and 6-keto-$PGF_{1\alpha}$, respectively.
Lipoxygenases are a group of iron-containing dioxygenases that catalyze the insertion of one oxygen molecule at carbon 5, 12, or 15 of arachidonic acid yielding 5*S*-, 12*S*-, or 15*S*-hydroperoxyeicosatetranoic acid (5-, 12-, or 15-HPETE) respectively. Further conversion of 5-HPETE by 5-lipoxygenase catalyzes the formation of 5-HETE and the unstable epoxide LTA_4 which is enzymatically hydrolyzed to LTB_4 or, upon addition of glutathione, converted to LTC_4. Removal of glutamic acid by γ-glutamyl transpeptidase generates LTD_4, and subsequent removal of glycine by a dipeptidase yields LTE_4. 12-HPETE and 15-HPETE can be converted into 12-HETE and 15-HETE respectively [129].

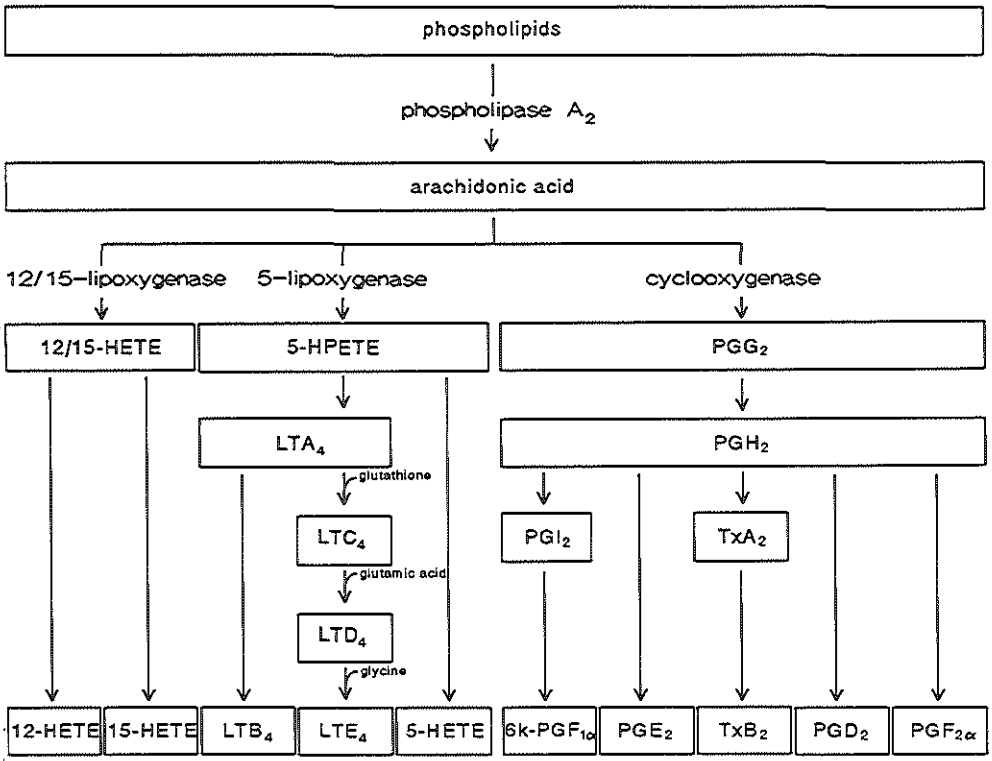


Figure 1.2. Schematic representation of metabolism of arachidonic acid from the phospholipid membrane into lipoxygenase and cyclooxygenase metabolites. *Abbreviations*: LT, leukotriene; PG, prostaglandin; HETE, hydroxy-eicosa-tetraenoic acid; HPETE, hydroxyperoxy-eicosa-tetraenoic acid; Tx, thromboxane.

cyclooxygenase metabolites

Almost all inflammatory cells are able to generate cyclooxygenase products, although the specific product varies from cell to cell. Human mast cells preferentially generate, upon stimulation with IgE, PGD_2 [124,166,229] whereas AM and airway epithelial cells predominantly generate PGE_2, $PGF_{2\alpha}$ and thromboxane [47,104,128]. Thromboxane and PGE_2 are the main arachidonic acid metabolites of secondary effector cells (eosinophils, neutrophils and platelets) [108,196,265].

The localization and classification of prostanoid receptors in the lung has been hampered by the fact that no specific antagonists are available. An alternative method for receptor typing is determination of a functional response upon challenge with eicosanoids [98] using such parameters, several subtypes of prostanoid receptors appear to be present throughout the respiratory tract. Thus, the most potent contractile response is mediated by thromboxane receptors [53,99] whereas prostacyclin receptors have been identified in guinea pig lung by measuring the PGI_2-induced response of adenylyl cyclase (see chapter 3).

Depending on the structure, prostaglandins alter bronchial muscle tone. $PGF_{2\alpha}$ and PGD_2 are much more potent bronchoconstrictors in asthmatic individuals than in normal subjects [219]. PGE_1 and PGI_2 relax human smooth muscle though their effect is small compared to that of isoprenaline. PGE_2 can either increase or decrease basal tone of isolated human airway smooth muscle preparations [97]. Apart from their effects on bronchial smooth muscle, cyclooxygenase products affect many different pulmonary inflammatory cells. PGE_2 has been shown to exhibit potent down-regulatory capacities for mainly macrophages, neutrophils and lymphocytes. Thus, it inhibits phagocytosis, mediator-production and cytotoxicity of macrophages, chemotaxis of macrophages and neutrophils and several lymphocyte functions [242]. Prostacyclin and thromboxane inhibit aggregation of platelets and neutrophils [266] whereas PGE_1 may inhibit neutrophil chemotaxis and adherence [45].

lipoxygenase metabolites

Arachidonic acid is metabolized via lipoxygenase into leukotrienes, lipoxins and several hydroxyacids (HETE's). Since the structural identification of slow-reacting substance of anaphylaxis (SRS-A, originally described by Brocklehurst,1960 [32]) as sulfidopeptide leukotrienes [26,191], these lipid mediators have been implicated in the processes of pulmonary inflammation [64].
Neutrophils, airway epithelial cells and AM predominantly produce LTB_4 whereas LTC_4, LTD_4, 5-HETE and other mono-HETE's are produced in only small quantities by these cells [26,82,118,179,105,127,221]. Eosinophils predominantly generate LTC_4, LTD_4 and 12-HETE [35,246] and 12-HETE is the primary lipoxygenase product of platelets [152]. Upon IgE-mediated stimulation, mast cells have been shown to produce LTB_4, LTC_4 and 5-HETE [178,211].

Specific binding sites for LTB_4 have been identified on human neutrophils. The identification of LTC_4 receptors has been hampered by the rapid transformation of LTC_4 into LTD_4. However, recent autoradiographic studies have mapped the distribution of LTC_4 and LTD_4 receptors in guinea pig lung [15]. In the human bronchi, such differentiation in LTC_4 and LTD_4 receptors appears to be absent [36]. Furthermore, it has been suggested that the majority of leukotriene receptors may reside in an intracellular pool which may be recruited to plasma membranes during activation [167].

The contractile effect of the sulfidopeptide-leukotrienes on human bronchial muscle has been extensively investigated [63,113,139]. Leukotriene C_4 and D_4 are approximately 1000 fold more potent than histamine in contracting human isolated bronchus [63]. LTE_4 is less potent than LTC_4 and LTD_4, but its effects are more prolonged. 5-and 15-HETE cause modest contraction of human bronchial muscle in vitro [56]. Leukotrienes B_4, C_4, D_4, and E_4 increase microvascular permeability at least 100 to 1000 times more effective than histamine [31,270]. LTB_4 is the most potent chemotactic and chemokinetic lipoxygenase product for neutrophils and monocytes in vitro [107,88] but is less effective for eosinophils [259]. This action is not shared by the sulfidopeptide-leukotrienes. LTB_4 also stimulates the release of lysosomal enzymes from macrophages and neutrophils [81], enhances the release of oxygen radicals from neutrophils [235] and enhances expression of surface complement (C3b) receptors on neutrophils and eosinophils [195]. Mono-HETE's are chemo-attractants for human neutrophils and eosinophils [106] whereas 5-and 12-HETE's and lipoxin A induce degranulation of human neutrophils [235,241].

platelet activating factor

Platelet activating factor was first recognized as a basophilic product capable of eliciting platelet activation [23]. Since, this mediator was termed platelet activating factor (PAF) and has been chemically characterized as an ether-linked phospholipid (1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine). PAF can be synthesized by two distinct pathways. The first is a two-step pathway which has been demonstrated in a number of inflammatory cells including macrophages [4], neutrophils [171], eosinophils [161] and platelets [24], which involves the production of the biologically inactive intermediate lyso-PAF by phospholipase A_2 (PLA_2). Further conversion of lyso-PAF into PAF requires a second enzyme (besides PLA_2) which is an acetyl

coenzyme A (CoA)-dependent acetyltransferase enzyme and probably the rate-limiting step for PAF-production by this pathway. A second pathway for PAF-synthesis involves cholinephosphotransferase, which produces PAF directly from ether-linked phospholipids [240]. This pathway may be required to maintain physiological levels of PAF for normal cell function whereas the rate-limiting acetyl transferase pathway is only activated in response to inflammatory stimuli [240]. PAF is also produced by basophils and mast cells although in these cells, the majority of the formed PAF does not appear to be a secretory product but is retained intracellularly [169,176]. Platelets and eosinophils produce PAF upon exposure to various chemotactic factors whereas AM synthesize PAF following allergen stimulation and phagocytic stimuli [8,161]. Epithelial cells have not been shown to generate PAF, but it has been suggested that these cells can produce PAF in an analogeous way to endothelial cells of the bronchial vasculature [183].

Research for the presence of receptors on tissues or cells requires the use of selective agonists and antagonists. Since a decade, several PAF-antagonists have been manufactured though the selectivity of these drugs remains questionable as some of the PAF-effects are completely antagonized whereas others are not affected. Alternatively, using radiolabeled PAF, PAF-receptors have been identified on platelets [252], neutrophils [253], and lung membranes [133].

PAF activates and has chemotactic activity for a wide range of inflammatory cells like platelets, eosinophils, neutrophils, monocytes and AM [259]. Thus, PAF induces the aggregation of platelets [23,256], neutrophils [89] and monocytes [272] and activates neutrophils, eosinophils and AM to produce arachidonic acid metabolites and oxygen radicals [35,115,200].

One of the most important properties of PAF is its ability to induce a non-selective and long-lasting increase in BHR in animals and man. Although PAF elicits BHR to a wide range of spasmogens (histamine, acetylcholine, serotonin, substance P), the increased responsiveness is not secondary to alterations in receptor number, affinity or post-receptor mechanisms with the exception of ß-adrenoceptors. In this respect, PAF has been shown to induce a down-regulation of ß-adrenoceptors in human lung tissues [3].

neuropeptides

Besides cholinergic and adrenergic innervation, a third neurological mechanism seems to be involved in the regulation of airway caliber and the modulation of pulmonary inflammatory responses. This is called the non-adrenergic, non-cholinergic (NANC) nervous system of which the neurotransmitters appear to be neuropeptides and include both inhibitory and excitatory compounds [13]. Epithelial damage causes capsaicin-sensitive C-fiber endings of the NANC-system to be exposed to mediators and irritants initiating the release of neuropeptides. In turn, these peptides may either contract or relax bronchial smooth muscle, cause microvascular leakage and mucus secretion and as such may play an important part in the pulmonary inflammatory responses.
Among the various neuropeptides (table 1.2.), substance P (SP) and vasoactive intestinal peptide (VIP) are present in the highest quantities in the lung and are therefore discussed in further detail. However, other neuropeptides my prove in future to be more important than SP or VIP.

Table 1.2. Neuropeptides of the airways.

substance P (SP)
vasoactive intestinal peptide (VIP)
peptide histidine isoleucine/methionine
neurokinin A
calcitonin gene-related peptide
neuropeptide Y
neuropeptide K
galanin
gastrin-releasing peptide
cholecystokinin octapeptide
somatostatin
enkephalins

substance P

Substance P (SP) is an 11 amino acid peptide, localized to nerves in the airways beneath and within the airway epithelium, around blood vessels and within airway smooth musles [175,250]. In addition, SP has been localized in eosinophils [5].

It has been postulated that the effects of SP are elicited by two distinct receptor subtypes. For the SP.P receptor (also known as the NK1-receptor), SP itself is the

most potent agonist whereas for the SP.E receptor (NK2-receptor), eledoisin (another tachykinin) is the most potent [17].

Substance P has been postulated to be involved in bronchial inflammatory responses. Besides its contractile effects on airway smooth muscle [173], it increases microvascular leakage [174], stimulates mucus secretion [54], increases the release of histamine from mast cells [84] and possibly amplifies neutrophil and eosinophil responses to chemotactic agents [206]. Furthermore, SP is chemotactic for monocytes and AM [117,222], stimulates oxygen radical- and lysosomal enzyme production from AM [19,116] and induces the production of TxB_2, PGE_2 and IL-1 from monocytes and AM [59,172].

vasoactive intestinal peptide

Vasoactive Intestinal Peptide (VIP) is a 28 amino acid which has been localized in (animal and human) lungs to neurones, ganglia, pre-ganglionic nerve terminals, nerve terminals in airway smooth muscle, around submucosal glands, and in bronchial and pulmonary vessels but not in airway epithelium. [68,155,249]. In addition, VIP can be produced by pulmonary inflammatory cells, such as mast cells, neutrophils and eosinophils [5,62,199].

VIP-receptors have been localized on a variety of different pulmonary cells by means of its ability to stimulate adenylyl cyclase to increase intracellular levels of cAMP. As such, VIP-receptors have been described for epithelial cells, submucosal glands, airway smooth muscle cells [159], platelets [58], monocytes [112], lymphocytes [66,201,202] and AM [223].

In addition to its bronchodilating activity, VIP has been shown to possess anti-inflammatory actions. In AM and monocytes, VIP decreases the respiratory burst and phagocytosis [170,257] and inhibits the antigen-induced histamine-release from mast cells [251], the PAF-induced serotonin-secretion and aggregation of platelets [58]. In T-cells, the release of IL-2 and proliferation are inhibited by VIP [151,203].
Although peptidergic sensory nerves are present throughout the respiratory tract [181] and, in theory, dysregulation of the NANC-system might lead to neurogenic and pulmonary inflammation (and subsequently to BHR), no clear role for neuropeptides has been shown in asthma or COPD. Thus, bronchial challenge of asthmatics with

either SP or VIP does not effect airway caliber (whereas neurokinin A causes bronchoconstriction) [205]. A possible explanation for this discrepancy might be the rapid degradation of these peptides by neutral endopeptidases (NEP)[111]. In this respect, it should be noted however, that the mechanisms of BHR induced by cigarette smoke, toluene diisocyanate and viral infections, are closely associated with neurogenic inflammation, as these inducers of BHR have been shown to decrease NEP-activity and increase SP-activity [75,135,238].

complement

Complement factors are not considered to belong to the mediators involved in pulmonary inflammatory reactions but they play an important role in host defense and in a number of pathological disorders of both immunological and non-immunological origin. The complement system comprises 17 plasma proteins which can be activated via two pathways: the classical components, which are the plasma proteins responsible for the lysis of cells bearing antibodies directed to cell surface antigens (designated as C1-C9) and the alternative pathway factors, which lead to a lytic terminal sequence without a requirement for antibody (designated as B, D and P) [33].

The biologic activities derived from complement activation stem largely from the cleavage of C3 and C5, resulting in the production of the so called anaphylatoxins C3a and C5a. C3a receptors have been identified on leukocytes, mast cells, and AM, while C5a receptors have been identified on mast cells, monocytes, platelets, and leukocytes [79,216].

One of the most widely studied effects of anaphylatoxins is their ability to induce activation of inflammatory cells. C5a has chemotactic activity for neutrophils [189], macrophages [217], basophils [145] and eosinophils [144] whereas C3a appears to be devoid of chemotactic activity [83]. In addition, C5a stimulates the release of lysosomal enzymes [182], free oxygen radicals [22], prostanoids, leukotrienes [49] and PAF [161].
Another important cleavage-product of C3 is C3b which binds specifically to the C3b-receptor (the immune adherence receptor) thereby facilitating the phagocytosis of opsonized antigens (antigens complexed to antibodies and complement).

The role of the complement system in asthma and COPD is still controversial as conflicting results have been reported. Some investigators described increased plasma concentrations of components of the complement system while others reported no change or even a decrease in their plasma levels [9,146,213].

1.3. concluding remarks

In the pathogenesis of asthma and COPD, pulmonary inflammatory reactions are clearly implicated. Typical characteristics (like BHR, excess mucus secretion, increased vascular permeability, edema formation, cellular infiltrates) result from the activation of inflammatory cells. Inflammatory mediators can be regarded as cross-talk signals between and activators of primary and secondary effector cells resulting in a cascade of inflammatory processes. Within this cascade, AM, by exhibiting its multiple actions, may act as modulators to control the mechanisms of inflammation. In chapter two, the function of AM within pulmonary inflammation (and subsequently in asthma and COPD) and its modulation by exogenous factors will be discussed in more detail.

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chapter two
the alveolar macrophage

2.1. introduction

The lung is continuously exposed to harmful foreign influences including infectious microorganisms, allergens, and toxic environmental agents potentially affecting homeostasis of the host. Respiratory disease(s) would therefore rapidly arise as a result of this persistent insult. However, adequate lung defense mechanisms consisting of the combined actions of mucociliary, phagocytic, and specific immune systems are present to maintain normal physiology of the lung.
The pulmonary alveolar macrophage, a member of the mononuclear phagocytes, occupies in this respect a unique position as it is a) among all pulmonary immune cells present in the largest amount, b) directly exposed to a relatively hyperoxic environment and c) in close contact with both airborne and blood-borne substances. Therefore, this cell type is intimately involved in maintaining the environment of the respiratory tract particle-free. While it was originally suggested that these cells served only a scavenger function by preventing antigens from entering the afferent arm of the immune response, it is now believed that they provide in addition an important function in regulating inflammatory- and immune responses in the lung.

2.2. terminology

Three types of macrophages can be recovered from the lung [24]. The alveolar macrophage (AM) is the cell type responsible for protection of the alveolar surface. The majority of these cells remain in the alveolar space without re-entering the alveolar wall [146]. A second pulmonary macrophage is the airway macrophage which is present in both large and small conducting airways. These cells may be present either as passengers on the mucous escalator, or may be found adhering to bronchial epithelium beneath the mucous lining. Airway macrophages probably represent the alveolobronchiolar transport of AM, although it has been suggested that these cells appear in the airways as a result of direct migration from the interstitial compartment through the bronchial epithelium [91]. The third kind of macrophage is the interstitial macrophage which is located in various connective tissue compartments of the lung [146] like alveolar walls, sinuses of lymph nodes and nodules and peribronchial and perivascular spaces.

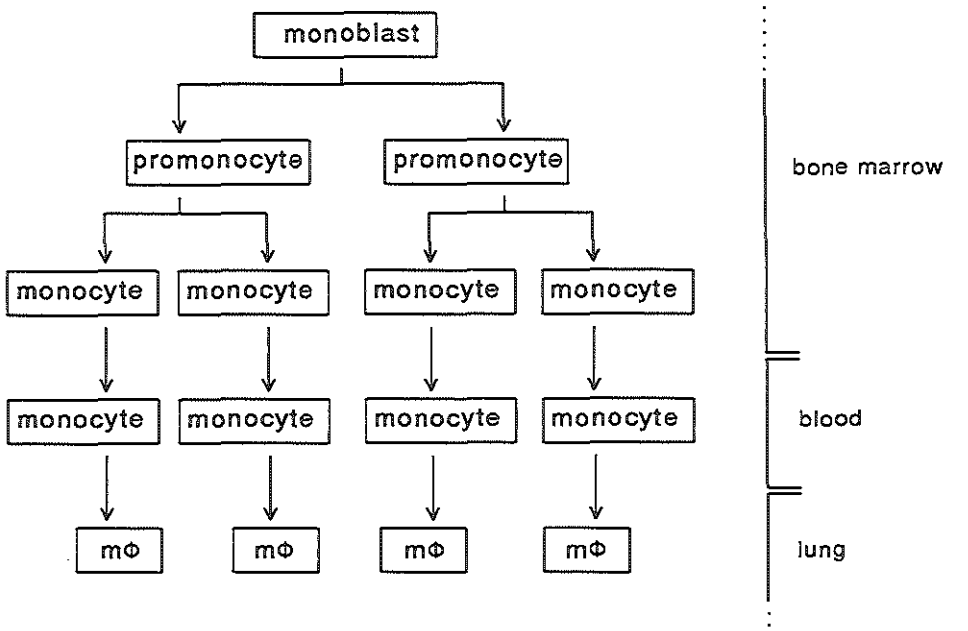

Figure 2.1. Schematic representation of ontogenesis of pulmonary macrophages

2.3. ontogenesis

Like other tissue macrophages which populate various organs of the body, pulmonary macrophages are derived from bone marrow progenitor cells (figure 2.1) [22,57,132]. Within the bone marrow, the monoblast divides into two promonocytes which in turn evolve into two monocytes each. Monocytes do not divide further, and leave the bone marrow within approx. 24 hours after formation. In normal steady state situations, the maintenance of the macrophage population depends on the influx of monocytes from the circulation and possibly on local division of interstitial macrophages. The eventual pulmonary origin of AM was suggested by Tarling who destroyed murine bone-marrow stem cells with ^{89}Sr. As a result, the number of circulating monocytes was severely depressed but the number of AM remained essentially unchanged [151]. Similarly, others have demonstrated that the number of AM remains unaltered during periods of monocytopenia induced by chemotherapy [61] or bone-marrow irradiation [134]. In addition, it has been shown that up to 70 % of the AM

population can be produced by inter-alveolar division of AM under normal steady state conditions [34] and that local thoracic irradiation does not affect the number and labeling index of circulating monocytes but reduces the number of labeled AM [151]. Nevertheless, it appears that during pulmonary inflammation (acute or chronic), proliferation of AM in the lung is minimal and that increases in the number of AM result mainly from the influx of monocytes into the lungs [23].

2.4. cellular functions of alveolar macrophages

Classically, cellular functions of AM are subdivided into two main categories: non-specific or inflammatory and more-specific or immunological functions (table 2.1). This classification should not be considered as an abstract division as both catagories are more or less interrelated p.e. antigen presentation (an immunological function) can only arise after phagocytosis (an inflammatory function). In the following paragraphs, the most important macrophage functions in host defense are discussed.

Table 2.1. Broad classification of alveolar macrophage functions into inflammatory and immunological functions.

inflammatory	phagocytosis
	respiratory burst
	enzyme production
	protein production
	mediator production
immunological	cytokine production
	antigen presentation
	tumoricidal activity

2.4.1. inflammatory functions

phagocytosis

Classically, the macrophage has been considered mainly as a phagocytic cell. In the lung, ingestion of inhaled microorganisms and other particles by AM constitutes an important first line of host defense. With some rare exceptions, opsonization of inhaled antigens is essential for phagocytosis to occur. Opsonization by immunoglobulins and complement factors (humoral opsonins) is the most important factor in promoting phagocytosis by AM [49]. A growing body of evidence suggests that human AM not only possess receptors for immunoglobulins but may have in

addition immunoglobulins bound to their surface. On human AM, in particular IgG_1, IgG_3, and IgG_4 have been documented [104]. Furthermore, a great portion of normal AM possess IgE-receptors. It has been shown that triggering of these receptors may lead to increased release of ß-glucuronidase, neutral proteases and oxygen radicals [81-83] and LTC_4 [122]. Human AM have also been shown to possess specific C3b-receptors [125].
Besides augmentation by humoral opsonins, the surfactant lining fluid of the alveoli might also enhance AM ingestion and -killing of microorganisms [110,129]. Its mechanism is not clear though it has been suggested that free fatty acids of surfactant, by enhancing surface tension, may serve as detergent, to increase the permeability of the cell wall for microorganisms [38].
After attachment to and recognition by the AM, antigens and microorganisms are phagocytosed via a complex process including partial invagination of the surface membrane and the formation of a phagosome which requires the arrangement of microfilaments, like actin and myosin [64].

respiratory burst

Closely associated with phagocytosis is the "respiratory burst", which generates reactive oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radicle ($OH\cdot$)[1]. The respiratory burst is an increase in cell metabolism initiated by phagocytosis which triggers a single enzyme system, NADPH or NADH oxidase, located on the plasma membrane. Activation of this system results in the reduction of oxygen to O_2^- which can be converted to H_2O_2 by superoxide dismutase (SOD). O_2^- and H_2O_2 may interact to form $OH\cdot$ and $^1O_2$. Moreover, in the presence of peroxidase, H_2O_2 may oxidize halides to oxyhalides, which are toxic to microbes and tumor cells and may cause peroxidation of cell membrane lipid components leading to cellular damage. Inhibitors of oxygen radicals are necessary to maintain tissue homeostasis. SOD catalyses the conversion of O_2^- to H_2O_2 which is degraded by catalase and glutathion peroxidase. These enzymes are present within the AM [1,64].

lysosomal enzymes

AM produce a variety of enzymes which are generally classified as lysozyme,

lysosomal acid hydrolases and neutral proteases. Though these enzymes function mainly intracellularly as digestive enzymes, they can be secreted into the extracellular environment, where they exert a variety of actions (p.e. the augmentation of microbial killing, the degradation of connective tissue, activation of the complement system, lysis of fibrin, etc. see table 2.2.).

Table 2.2. Enzymes secreted from alveolar macrophages and their main function.

enzyme	function
lysozyme	bactericidal
neutral proteases	
plasminogen activators	lysis of fibrin
collagenases	cleavage of collagen
elastase	proteoglycan degrading
angiotensin converting enzyme	conversion of angiotensin I→II
acid hydrolyses	
proteases	cleavage of proteins
lipases	hydrolysis of triacylglycerol
desoxyribonucleases	degradation of DNA
phosphatases	cleavage of phosphated substrates
glycosidases	hydrolysis of glycosidic bonds
sulfatases	cleavage of sulfhydryl bonds
arginase	antimicrobial, tumoricidal
lipoprotein lipase	cleavage of lipoproteins

Lysozyme, the major secretory product of macrophages [62] is bactericidal for a significant number of organisms by cleaving the ß-1,4-bonded disaccharrides of bacterial cell walls [76]. Besides by phagocytic stimuli [36], the secretion of this enzyme can be augmented by immunological stimulation [66,90] and smoking [69].
More specifically, AM contain large amounts of lysosomal acid hydrolases which are stored intracellularly and can be selectively released in response to specific stimuli. Their activity in the extracellular environment is limited to neutral pH. Under certain conditions, when pH of the environment decreases (like in acute inflammation), acid hydrolases affect the integrity of collagen, basement membranes and other components of connective tissue [108].
The neutral proteases, in particular elastase, collagenase, and plasminogen activator, are, by degrading connective tissue components of the lung, postulated to be important factors in the pathogenesis of certain chronic lung diseases [140,141].

plasma proteins

AM may release plasma proteins, like complement factors [19,53], coagulation factors [112], fibronectin [4], transcobalamin II [118], α_2-macroglobulin [73], and apolipoprotein E [16] which participate in inflammation, tissue repair, immunoregulation, and molecular transport (table 2.3).
Complement factors promote phagocytosis by acting as opsonins and participate in lysing foreign cells by the formation of C5-9-complexes. In addition, these pro-inflammatory mediators (C3 and C5) may serve as chemotaxins.
Coagulation factors contribute in tissue repair and immunologically induced tissue injury.
Apolipoprotein E participates in the transport of cholesterol and triglycerides to the liver [16]. In addition, this compound has immunoregulatory functions, in that it can inhibit T-cell function [40] and the phytohemagglutin-enhanced incorporation of ^{32}P into lymphocyte phospholipids [9].

Table 2.3. Plasma proteins produced by alveolar macrophages and their main functions.

protein	function
complement factors	opsonization, chemotaxis
coagulation factors	tissue repair (coagulation)
transferrin	iron transport
transcobalamin II	vitamine B_{12} transport
apolipoprotein E	immunoregulation
fibronectin	tissue repair (collagen-cell interaction)
α_2-macroglobulin	proteinase inhibitor

arachidonic acid metabolites

Arachidonic acid liberated from the phospholipid membrane is converted via two major enzymatic pathways: a) the cyclooxygenase pathway resulting in prostaglandins and thromboxanes and b) the lipoxygenase pathway generating leukotrienes and HETE's. Most of these metabolites (eicosanoids) have been implicated in pulmonary inflammatory processes (described in chapter one).

In AM prostaglandin E_2 and thromboxane A_2 are the main products of the cyclooxygenase pathway whereas PGD_2, $PGF_{2\alpha}$ and hydroxyheptadecatrienoic acid (HHT) are generated to a lesser extent. These cells also produce a variety of

lipoxygenase metabolites i.e. LTC_4, LTD_4, LTE_4, 5-,12-, and 15-HETE but predominantly LTB_4. Which arachidonic acid metabolite is preferentially produced and released depends, besides on the isolation procedure of the AM, strongly on the incubation conditions and the stimulating agent. In this respect, several conflicting results have been reported. Using AM which were purified and incubated under culture conditions, the calcium- ionophore A23187 specifically induces mainly the production of lipoxygenase products [14,25,29,51,89,97,136] while others reported the release of PGE_2, PGD_2 and TxB_2 (the stable metabolite of TxA_2) in response to this stimulus [11,13,14]. Lipopolysachacharide (LPS) induces, in contrast to A23187, mainly the release of cyclooxygenase products [25], whereas zymosan (a phagocytosis stimulus) induces the production of both cyclooxygenase and lipoxygenase metabolites [14,88,136].
A possible explanation for this discrepancy in the preferential production of eicosanoids could be that in most of the studies (both human and animal), arachidonic acid metabolites have been determined in AM which were cultured, previously to (for purification from other cells) or during the incubation period, in plastic culture disks. This incubation procedure can be regarded as an adherence-, or even better, as a phagocytosis stimulus ("frustrated phagocytosis"). Indeed, conformational changes of the membrane during phagocytosis have been shown to induce the release of arachidonic acid metabolites (both cyclooxygenase and lipoxygenase products) [85]. Alternatively, the calcium-ionophore A23187 stimulates AM to produce preferentially lipoxygenase products, possibly via a calcium-dependent mechanism as it has been shown that lipoxygenase, in contrast to cyclooxygenase, is a Ca^{2+}-dependent enzyme [75].

platelet activating factor

The biosynthesis of platelet activating factor (PAF) shows an important similarity to the biosynthesis and metabolism of arachidonic acid as a Ca^{2+}-dependent phospholipase A_2 is necessary for both compounds to be liberated from the phospholipid membrane [143]. Indeed, it has been shown that AM produce PAF upon stimulation with A23187 or zymosan [3,5].

2.4.2. immunoregulatory functions

AM participate in pulmonary immune reactions in several ways. First, AM have been shown to serve as effectors for cell-mediated immune reactions: particulate antigens interact with specifically sensitized T-lymphocytes in the lung resulting in local elaboration of lymphokines which activate AM. Activation in this context leads to enhancement of phagocytosis and microbicidal activity, particularly with respect to viruses, fungi, and intracellular bacteria. Second, AM may initiate and modulate the induction phase of both humoral and cellular immune response: AM ingest particulate antigens, process and present them on their surface membranes to specific antigen-reactive B- and T-lymphocytes, inducing the proliferation and differentiation into effectors of humoral and cell-mediated immunity respectively. In this role, AM might enhance or suppress the expression of the pulmonary immune reactions.
It has been suggested that AM-populations consist of different functional subpopulations; some of which enhance while others strongly suppress the immunological response [15,43]. In addition, it has been postulated that lymphocyte proliferation induced by AM depends largely on the macrophage-to-lymphocyte ratio: low ratios enhance and high ratios suppress lymphocyte proliferation [46,167].
Thus, the ultimate balance of enhancing or suppressive actions of AM is modulated by the diversity of the macrophage population. Theoretically, this implicates that immunological action of AM subpopulations depends on factors like macrophage maturation and *in vivo* macrophage activation by immune or inflammatory processes.

cytokines

AM can play an important role in the pulmonary immune responses via the production of a variety of cytokines (monokines and other mediators of cellular immunity). Macrophages are capable of secreting a variety of cytokines (table 2.4.) of which interleukin 1 (IL-1) and tumor necrosis factor (TNF-α) are produced in the greatest amounts [84,139]. Other cytokines produced by AM include platelet derived growth factor (PDGF) [98,138], α-interferon (IFN) [47], γ-IFN [130], fibroblast growth factor (FGF) [12], colony stimulating factor (CSF) [103], macrophage inflammatory protein (MIP-1) [165], IL-6 [80], transforming growth factor (TGF-ß) [7] and insulin-like growth factors (IGF-I) [133].
Interleukin-1 (identified in two forms: IL-1α and IL-1ß with, as yet, no difference in

biological activities) modulates accessory growth factor activity for T-cells, chemotaxis of polymorphonuclear leukocytes, macrophages and lymphocytes. Tumor Necosis Factor-α (TNF-α) is, unique as it is, extremely toxic. It is cytotoxic for a variety of tumors, has variable growth activity for fibroblasts [149], modulates growth and proliferation of B-lymphocytes [78], can cause severe tissue damage [124,158] and induces the production of IL-1, prostaglandins [10] and PAF [28].

Table 2.4. Cytokines produced by alveolar macrophages and their main actions*.

cytokine	function
IL-1	proliferative, mitogenic, chemotactic
TNF-α	necrotic, toxic (tumors)
α-IFN	antiviral
γ-IFN	antiviral
PDGF	mitogenic, chemotactic
FGF	mitogenic, chemotactic
CSF	chemotactic
MIP-1	chemotactic, pro-inflammatory
IL-6	stimulative for protein synthesis (B cells, liver)
TGF-ß	mitogenic, chemotactic, immunosuppressive (T and B cells)
IGF	proliferative

* *abbreviations* are clarified in text.

Platelet Derived Growth Factor (PDGF) is a highly cationic glycoprotein of 30 kDa which can act on neutrophils (stimulation of phagocytosis and chemotaxis and blockade of the respiratory burst) [164], fibroblasts and vascular smooth muscle cells. Interleukin-6 (or interferon $ß_2$, B-cell differentiation factor) acts on B-cells to enhance immunoglobulin production [18].

antigen presentation

One of the most important functions of AM in the respiratory immunological defense mechanisms is antigen presentation. Antigen-specific activation of T-cells requires the antigen to be presented by an accessory cell in an immunologic form. To do this, the presenting cell must present the antigen to the lymphocyte in relation to a specific antigen on its own plasma membrane. These surface molecules (HLA-antigens in humans, H-2-antigens in mice) are expressed by the majority of AM implicating them as an important antigen-presenting cell [71,100]. In studies of antigen presentation, the

ability of AM to present antigen is often compared to peripheral blood monocytes. In this respect, several conflicting results have been reported. Thus, Ettensohn and Roberts [50] and Toews *et al.* [155] showed inferior while Laughter *et al.* [87] described a superior ability of AM to present antigen compared to blood monocytes.

antitumor activity

Mechanisms involved in host resistance against malignant tumors have been found to be mediated mainly by cellular effectors including killer T-cells, natural killer cells, lymphokine activated killer (LAK) cells and macrophages. There are several mechanisms by which AM kill tumor cells. Thus, extracellular release of O_2^- [105], protease [2], C3a [52], arginase [39] and a variety of as yet unidentified tumor cytotoxic or cytostatic factors [27,137] are associated with tumoricidal actions of AM. However, more recent evidence suggests that direct macrophage-tumor cell contact is the main phenomenon required for macrophage antitumor activity whereas the release of several tumoricidal factors results from cell-cell contact [2].
Tumoricidal activity of AM can be augmented by LPS [144,145,161], liposomal presented muramyl dipeptide (DMP) [145], phorbol myristate acetate (PMA) [107] and by drugs which interfere with the antioxidant enzymatic system of the tumor cell (favouring a role of oxidative mechanisms in this activity) [108].

2.5. alveolar macrophages in asthma and copd

The recently developed technique of fiberoptic bronchoscopy has enabled to investigate macrophage function more properly in asthma and COPD. However, differences in methods of isolation, incubation and assessment of functional activity as well as the variation in subjects, hamper to determine the exact role and nature of AM.
It has been shown that the number of macrophages in BAL is increased after allergen challenge, probably as a result from migration from the peripheral monocyte compartment [102]. In allergic and aspirin-sensitive asthmatics, the viability of AM, the phagocytosis of non-opsonized zymosan as well as the production of cyclooxygenase metabolites are all impaired [60]. Balter *et al.* showed no alterations in eicosanoid metabolism (either unstimulated or A23187-stimulated) among AM from controls, atopic and asthmatic subjects [13] whereas other investigators noted marked

increased production of lipoxygenase metabolites (e.g. LTB_4, LTD_4, and 5-HETE) in AM from asthmatics and COPD patients as compared to controls [30,41,42]. In addition, AM from asthmatics, in contrast to control AM release PAF when stimulated with specific allergens [6]. The IgE-dependent or opsonized zymosan-induced production of oxygen radicals [33] and ß-glucuronidase [83,102,156,157] is also enhanced in AM from asthmatics, which suggests that macrophage phagocytosis-related secretory processes are stimulated in AM from asthmatics.
In contrast to this, the accesory cell (immunological) functions of AM seem to be impaired in asthmatics. Thus, unstimulated IL-1 release is increased in AM from asthmatics when compared to controls, whereas upon LPS-, allergen-, or anti-IgE-stimulation a decreased IL-1 production is reported [63,117]. Furthermore, AM from asthmatics exhibit a decreased suppressor cell activity when compared to controls [8]. Gosset *et al.* suggested that impaired IL-1 release in these cells results from an increased production of an IL-1 inhibitory factor [63].

AM secrete a variety of elastases and proteinase inhibitors which can specifically be inactivated by oxygen radicals or metalloenzymes. It has been shown that AM from smoking COPD patients secrete more elastases and exhibit higher elastolytic activity than AM from normal smokers [74,101,131]. Furthermore, AM from smokers produce an inactive form of α_1 proteinase inhibitor [77]. These data imply an important role for AM in the pathogenesis of emphysema associated with the imbalance between neutrophil elastase and α_1 proteinase inhibitor.

2.6. exogenous modulation of alveolar macrophages

2.6.1. effect of drugs

The drugs used in the treatment of both asthma and COPD can be devided into mainly two categories, bronchodilators and anti-inflammatory drugs.
Bronchodilators act mainly by reversing contraction of airway smooth muscle, although many have additional properties which may prove to be beneficial in therapy (see below). Three classes of bronchodilator drugs are currently used: beta-adrenergic agonists, theophylline-derivatives and anticholinergics.
Beta-adrenergic agonists exert their effects on bronchial smooth muscle through interaction with $ß_2$-adrenergic receptors which results via increased concentrations

of intracellular cyclic AMP to relaxation of the smooth muscle cells. An additional property of ß-adrenergic agonists is the prevention of IgE-stimulated mediator-release from lung mast cells [159] and neurotransmitter release from cholinergic nerves [127].
Besides bronchodilation, several other effects of theophylline and its derivatives have been proposed to be of therapeutic importance in asthma and COPD: increase in mucociliary transport, inhibition of mediator-release and suppression of edema-formation [150]. Although its precise mode of action is still unknown, several cellular mechanisms of action have been suggested including inhibition of cyclic AMP- and cyclic GMP- phosphodiesterase [20,115], adenosine antagonism and the release of catecholamines [68]. Cholinergic antagonists, like ipatropium bromide, inhibit vagal reflex bronchoconstriction.

Since the pathogenesis of asthma and COPD is clearly associated with inflammation, anti-inflammatory drugs are frequently used in the treatment of both diseases to suppress the inflammatory processes. Drugs in this category include corticosteroids, disodium cromoglycate (DSCG) and nedocromil.
The anti-inflammatory actions of corticosteroids result mainly from their inhibitory effect on mediator-release from a variety of cells. Corticosteroids inhibit the complete arachidonic acid metabolism by suppressing the release of arachidonic acid from phospholipids. This effect is mediated by a protein, called lipomodulin, which blocks the action of phospholipase enzymes [21,70]. An important additional effect of corticosteroids, especially in asthma and COPD, is their action on beta-adrenoceptors. In neutrophils and lymphocytes, these drugs increase receptor-density and receptor-affinity as well as the coupling of the receptor to adenylyl cyclase [44,45]. This means that corticosteroids attenuate the desensitization that stems from internalization of receptors and uncoupling of the receptors from adenylyl cyclase [147,160]. Desensitization is the reduced ability of a receptor-mediated transduction-system to respond to receptor stimulation as a result of repeated exposure of the receptor to agonists (chapter three). This phenomenon might contribute to the distinct beneficial effects of corticosteroids in the treatment of asthma and COPD.
DSCG, nedocromil amd ketotifen have been shown to inhibit mediator-release from pulmonary cells (mainly mast cells) [32,48,97] whereas ketotifen in addition has been shown to inhibit PAF-induced bronchoconstriction [114]. Their mechanisms of action are still poorly understood but probably involves the stimulation of surface-receptors.

With the exception of corticosteroids, the effects of drugs on functional aspects of AM are still poorly documented. Beta-adrenergic agonists have been shown to have little or no effect on arachidonic acid metabolism [56], to decrease ß-glucuronidase-release [82] and to be able to increase [109] as well as decrease [37,67] oxygen radical and H_2O_2 production. Theophylline decreases the bactericidal activity and oxygen radical production of AM [111] whereas disodium cromoglycate has been shown to inhibit the allergen-induced release of ß-glucuroninase from both passively sensitized normal AM and AM from astmatics [82].
Corticosteroids have been shown to inhibit most inflammatory and immunologically related features of AM. Thus, phagocytosis, intracellular killing, production of various enzymes (elastase, collagenase, ß-glucuronidase) and oxygen radicals are all markedly inhibited by corticosteroids [64,82,95,135,162]. In addition, corticosteroids inhibit metabolism and secretion of arachidonic acid metabolites [55,116,117], and cytokines, like IL-1 [142], TNF-α [148] and GM-CSF [86].

2.6.2. effect of smoking

Cigarette smoking is concidered to be a major risk factor for the development of COPD [118]. Considering the evidence presented in this chapter for an important role of AM in the defense of the respiratory tract in both normal and pathophysiological circumstances, putative adverse effects of smoking on a variety of functional aspects of this cell has been extensively studied. Smoking results in an increase in the number of AM recovered by BAL whereas the viability of the cell pupulations remains unaffected [56]. AM from smokers appear to be structurally changed in that they are enlarged as a result of an increase in vesicles containing lipids, proteins and pigment particles [31]. In addition, alterations in the structure and size of the cytoplasmatic reticulum, Golgi-apparatus and mitochondria have been observed [122].
Phagocytic capability of AM from smokers has been shown to be increased [96] or decreased [54] whereas most investigators reported a similar phagocytic capability of AM from smokers and nonsmokers [79,94,123,125,152]. This discrepancy is probably due to a number of differences in experimental design like variations in cellular culture, human subjects or phagocytic stimuli. The release of lysosomal enzymes and reactive oxygen species is generally increased in AM from smokers [17,65,72,81,92] and has been regarded a cooperative mechanism in the induction of emphysema [128].
From these data, it appears that although the phagocytic capability is not affected,

phagocytosis related features of AM (production of lysosomal enzymes and oxygen radicals) is increased in smokers, probably as a result of constant exposure to cigarette smoke. Accessory cell-functions of AM which include the production of cytokines, antigen presentation and cytotoxic- and tumoricidal activity appear to be impaired in smokers AM. Thus, IL-1-release and cytotoxic activity towards various allogeneic target cells are inhibited in AM from smokers [26,153,166].
Finally, the production of arachidonic acid metabolites of both cyclooxygenase and lipoxygenase pathways is impaired in AM from smokers [61,88,89,93,162].

2.7. concluding remarks

As can be concluded from the information presented in this chapter, AM play a predominant role in the host-defense of the respiratory tract in both normal and pathophysiological conditions by exhibiting typical non-specific (inflammatory) and specific (immunological) properties. In both asthma and COPD, phagocytosis-related secretory functions (e.g. production of oxygen radicals and lysosomal enzymes) are enhanced whereas accessory cell functions (e.g. IL-1 production and suppressor cell activity) are impaired. This tendency of AM towards enhanced primary effector functions and impaired accessory cell functions can also be observed in AM from smokers when compared to nonsmokers. Thus, concluded only from *in vitro* studies, smoking once again can be regarded as an additional risk factor for both asthma and COPD. Although literature data are scarce, drugs used in the treatment of asthma and COPD seem to affect some of the macrophage functions (mainly its primary functions). An impairment of most AM functions is obtained when corticosteroids are applied. Whether this can be regarded as beneficial in the treatment of asthma and COPD remains to be elucidated when the role of cellular interactions and immunological functions of AM in the pathogenesis and pathophysiology of both diseases are established.

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chapter three
modulation of cellular activity

3.1. introduction

In order to respond and adjust to external information, cells are equipped with membrane structures which are referred to as transmembrane signalling systems. The transfer of external signals into metabolic changes via formation of so called second messengers was originally supposed in 1956 by Sutherland and co-workers [98] but not until 1971 described in further detail by Rodbell *et al.*[104]. They showed, using the enzyme adenylyl cyclase (AC) which converts ATP into the second messenger cyclic AMP (cAMP), that certain hormones produce their intracellular effects through interaction with a GTP-binding transducer. This led to the concept of a transmembrane signalling which comprises three components: receptors which specifically bind hormones, a GTP-dependent transducer and an effector which is activated to produce a second messenger (an intracellular chemical signal) through interaction with the transducer (figure 3.1.).

Furthermore, the GTP-binding site of the tranducer might also be a GTPase since GTP analogs (unhydrolyzable) stimulate AC in the absence of hormones [19]. In addition, it was shown that GTP-hydrolysis serves as a turn-off mechanism in the activation of AC and that GDP remains bound to the system thus holding it in an inactive state. Receptors were thought to activate the system by promoting the release of GDP, a concept which led to the formulation of the GTP regulatory cycle model (figure 3.2.).

3.2. G-proteins

The transducer was called a G-protein, a term derived from guanine nucleotide binding protein [106]. Later on, G-proteins were found to be multimeric proteins (heterotrimers) composed of α, ß, and γ subunits [56,93].
Differences in size, structure and function of the α-subunit presently serve to distinguish the various G-proteins (table 3.1). G_s is generally involved in the stimulation of AC whereas G_i is thought to be inhibitory for this enzyme. G_o is implicated in the regulation of Ca^{2+} and K^+ channels and G_t (or transducin) is the G-protein involved in visual transduction and stimulation of cyclic GMP-

phosphodiesterases (cGMP-PDE). Finally, G_{PLC} is associated with the stimulation of phospholipase C [18].

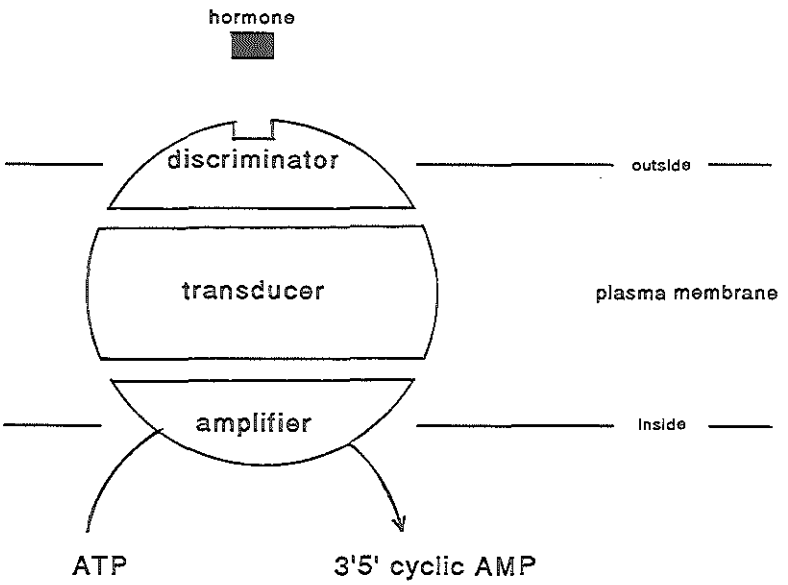

Figure 3.1.: Schematic representation of the signal transducing system as originally proposed by Rodbell *et al.* The discriminator represents the hormone receptor, the amplifier is the catalyst of adenylyl cyclase which converts ATP into 3'5' cyclic AMP (adapted from Rodbell,1971[104])

The α subunit contains a single, high-affinity binding site for guanine nucleotides and possess the GTPase activity which is crucial for the termination of the signal. This subunit also contains the site(s) for NAD-dependent ADP-ribosylation catalyzed by bacterial toxins. The α subunit of G_s ($G_{s\alpha}$) can be ADP-ribosylated by cholera toxin whereas the α subunits of G_i and G_o ($G_{i\alpha}$ and $G_{o\alpha}$ respectively) can be ADP-ribosylated by pertussis toxin. ADP-ribosylation causes specific alterations in the amino acid sequences of the α subunit resulting in the inhibition of the intrinsic GTPase activity and the subsequent accumulation of the α subunit in the GTP-liganded state (see below) [18].

The ß and γ subunits of G proteins are closely associated with each other. Their

function is as yet poorly defined though it has been suggested that the ßγ complex may participate in anchoring the α subunit to the plasma membrane [123].

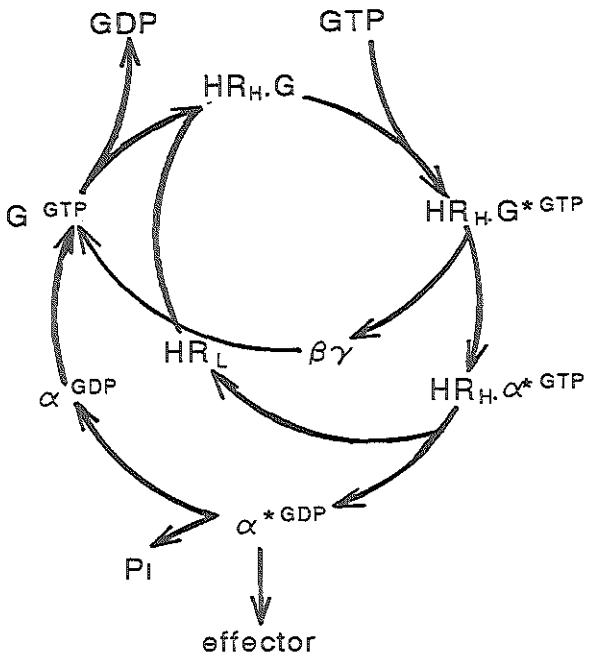

Figure 3.2. The mechanism of transduction by G proteins. The low-affinity form of the receptor (HR_L) interacts with the GDP-liganded heterotrimeric G protein (G^{GDP}) to form the high-affinity state of the receptor (HR_H) as a hormone receptor-G protein complex ($HR_H.G$). Occupation of HR_H by hormones activates the receptor to stimulate the release of GDP. GTP then binds to the nucleotide site on the α subunit of the G protein, promoting the release of the G protein from the hormone receptor and the dissociation of the G protein ßγ complex from the α subunit. The GTP-liganded and active α subunit (α^{*GTP}) then modulates the activity of the effector. The GTP is hydrolysed by the intrinsic GTPase activity to form α^{GDP}, which has high affinity for the ßγ complex and associates with it to form the heterotrimeric resting state of the G protein (G^{GDP}). The hormone receptor, upon dissociation of α^{*GTP}, returns to the low affinity state (HR_L) until it encounters another G^{GDP}.

Although heterogeneity exists amongst ß and γ subunits [33,39,128], the ßγ subunit

complexes of G_s, G_i and G_o can be interchanged. As receptor-mediated activation of G proteins requires both α and $\beta\gamma$ subunits, which are heterogeneous, it has been suggested that each class of receptors might recognize its own specific $\alpha\beta\gamma$ structure [18].

Table 3.1. G-proteins and their effector enzymes

G-protein	effector enzyme
G_s	adenylyl cyclase (stimulatory)
G_i	adenylyl cyclase (inhibitory)
G_o	ionic channels
G_t	cGMP-phosphodiesterase
G_{PLC}	phospholipase C

3.3. effector enzymes and second messengers

In order to respond to a variety of external stimuli, cells possess a large number of specific receptors. About 80 % of all known receptor-stimuli (hormones, neurotransmitters, autocrine and paracrine factors) elicit their cellular responses through the interaction with G proteins which activate only a small number of effector systems. In addition, though the primary messengers (receptor-stimuli) are many, the number of distinct receptors that mediate their action is even larger. Each primary messenger may bind to several subtypes of receptors, which in turn may interact with distinct effector systems. In short, an enormous number of receptors are coupled to only 7-8 known effector enzymes.
As mentioned in part 3.1., effector systems are generally membrane-bound enzymes which, upon interaction with G proteins, produce second messengers which in turn modulate cellular activity. The most important effector systems in the transmembrane signalling systems are discussed below, with special emphasis on AC.

3.3.1. phospholipase C

Upon G protein interaction, phospholipase C (PLC) stimulates phosphatidyl inositol (PI) turnover. In this PI-cycle, phosphatidyl inositol 4,5-biphosphate ($PI_{4,5}P_2$) is formed by a two-stage phosphorylation of phosphatidyl inositol (PI). Through the action of phosphomonoesterases $PI_{4,5}P_2$ can be converted back to PI [10]. This cycle is interrupted when PLC is activated which leads to the cleavage of $PI_{4,5}P_2$ into inositol

1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) [9]. IP_3 is the second messenger capable of modulating the intracellular Ca^{2+} levels by promoting its release and transport from internal stores (mobilization) [10,57,81]. DAG has multiple actions as a second messenger including protein kinase C activation (and consequently protein phosphorylation)[92] and phospholipase A_2 activation (and consequently release of arachidonic acid). In addition, DAG can be cleaved by diacyl- and monoacylglycerol lipases resulting directly to the liberation of arachidonic acid [80].

3.3.2. Ca^{2+} and K^+ channels

G proteins mediate effects between membrane receptors and ionic channels in two ways: indirectly after activating membrane associated effector enzymes which act via second messengers on ionic channels, and directly by activating ionic channels.
The indirect modulation of the activity of ionic channels by second messengers has been demonstrated for cGMP, cAMP, IP_3 and DAG. Thus, cGMP keeps Na^+ channels open [126], but when hydrolyzed by light-activated cGMP-PDE the cGMP content is decreased which results in the closing of these channels and the hyperpolarization of the membrane [30,55]. An elevation in intracellular cAMP concentrations, through AC stimulation, activates protein kinase A (see below) which in turn promotes the phosphorylation of Ca^{2+} channels and consequently causes the opening of the channels [31]. In analogy, DAG, formed through PLC activation, activates protein kinase C which may phosphorylate K^+ and Ca^{2+} channels [26]. IP_3 releases Ca^{2+} from internal stores which can alter the activity of a number of membrane channels, in particular Ca^{2+}-activated K^+- and nonselective cation channels [9].
Direct modulation of ionic channels involves a G protein distinct from the G proteins associated with AC (G_s/G_i), cGMP-PDE (G_t) or PLC (G_{PLC}). Using voltage clamp techniques, it was shown that activation of K^+ and Ca^{2+} channels were GTP-dependent processes, distinct from the known GTP-dependent processes, which could be mimicked with GTP analogs (Gpp(NH)P or GTPγS) and blocked with pertussis toxin [87,75,96,113].

3.3.3. cyclic GMP phosphodiesterase

Cyclic guanine-3',5'-monophosphate (cGMP) is a nucleotide formed through activation of guanylyl cyclase which exists in both cytoplasmatic and plasma

membrane bound configurations [111]. Although guanylyl cyclase activity is regulated by hormones, neurotransmitters and other agents, it differs from AC in that the former is not activated through G protein interactions [47]. The soluble form (cytoplasm located) can be specifically activated by free radicals and nitrovasodilators (like sodium nitroprusside) [87] whereas the membrane bound form is probably activated through receptors (like atrial natriuretic peptides or ANP) [52,135,139].
In addition, it has been proposed that PI-turnover is associated with guanylyl cyclase activity, probably via increased Ca^{2+} levels, as agents which stimulate PI-turnover may elevate intracellular concentrations of cGMP [8,86]. As cGMP concentrations are increased trough activation of guanylyl cyclase, a decrease in cGMP is accomplished by activation of a cGMP-specific phosphodiesterase (cGMP-PDE) which is a G protein consisting of an α, ß, and γ subunit [44]. In rods and cones this G protein is also called transducin, hence its name G_t [126]. In these cells, the α subunits may differ slightly, one form being expressed primarily in rods, the other in cones [74].

3.3.4. adenylyl cyclase

Cyclic adenosine-3',5'-monophosphate (cAMP) is a nucleotide formed from cellular ATP through activation of a membrane bound enzyme called adenylyl cyclase (AC). Cyclic AMP is metabolized into 5'-AMP by phosphodiesterases. The transmembrane signalling system of AC comprises the three major components (i.e. outer-membrane bound receptors, coupling G protein and inner-membrane bound catalytic moiety AC) [104]. The ß-adrenoceptor coupled AC system is regarded as one of the most important systems for our understanding of transmembrane signalling [38,73]. Beta-adrenoceptors are coupled via G_s proteins to stimulate AC and increase intracellular levels of cAMP. This G_s protein can be specifically ADP-ribosylated with cholera toxin (CT) leading to persistent activation of AC. A direct activation (without involvement of G_s) of AC is obtained with the diterpene forskolin which directly binds to a site on the catalytic moiety which is distinct from the G_s binding site [43,114].
Inhibitory effects of guanine nucleotides on AC were recognized even before stimulatory effects [22]. Using pertussis toxin which ADP-ribosylates a 41 kDa protein (distinct from cholera toxin substrates), G_i was identified as a G protein responsible for the inhibition of AC [132]. The mechanism by which agonists induce an inhibitory signal to AC still remains to be elucidated. Thus, although the overall effect of receptor-coupling to G proteins is comparable to other G protein-linked systems

(dissociation of the α subunit from the $\beta\gamma$-complex), both $G_{i\alpha}$ and $G_{i\beta/\gamma}$ (even more than $G_{i\alpha}$) have been reported to inhibit AC directly [65].
Summarizing, AC can be specifically stimulated via interaction with G_s (coupled to 'stimulatory' receptors like ß-adrenoceptors) and inhibited via G_i (coupled to 'inhibitory' receptors like α-adrenoceptors).

3.4. second messengers and intracellular events

Having dealed in the former section with how the information of primary messengers (hormones, neurotransmitters, mediators, etc.) is transformed via complex transmembrane signalling systems into second messengers, the question that needs to be addressed is how these second messengers regulate the activity of the cell.
IP_3 acts as an intracellular messenger to mobilize calcium from an internal, nonmitochondrial pool [9]. Other products of PI-breakdown are slightly or not effective in this respect [61]. It has been suggested that IP_3 exerts its action through a specific receptor for IP_3 in the intracellular calcium pool [122]. When a critical concentration is reached, Ca^{2+} activates calmodulin which in turn activates specific protein kinases (including protein kinase C) leading to protein phosphorylation [9,92].
Whereas Ca^{2+} can activate different kinases, DAG specifically activates protein kinase C which leads to phosphorylation of proteins [64,92]. In addition, activation of protein kinase C may result in diminished release of intracellular Ca^{2+} [68], inhibition of phospholipase C activity [13] and inactivation of IP_3 [89] suggesting a possible feedback mechanism between IP_3 and DAG. One strong argument against the existence of such a feedback mechanism is the fact that both second messengers act synergistically to enhance arachidonic acid liberation: IP_3 by mobilizing intracellular Ca^{2+} which can activate phospholipase A_2 (leading to the liberation of arachidonic acid) [70] and DAG via direct conversion by diacylglycerol lipases [80].
The underlying intracellular mechanisms associated with cyclic GMP as a second messenger are still a subject of debate. It has been suggested that cGMP interacts with some specific phosphodiesterases to stimulate the breakdown of cAMP [7]. Most of the reported studies have been performed using the light-sensitive cones and rods of the retina [58,102] whereas there is very little information about the existence of cGMP-PDE in other isolated cell types.
Cyclic AMP activates protein kinase A which consists of a catalytic subunit (C) and regulatory subunits (R) [32,69,77]. These regulatory subunits appear to exist in two

different forms, designated as R_I and R_{II}. Binding of cAMP to protein kinase A results in the dissociation of the regulatory and catalytic subunit. The catalytic subunits (C) become active to phosphorylate protein hydroxy-amino acid sequences whereas R_I and R_{II} may transport cAMP to the nucleus. These R-cAMP complexes are believed to couple to DNA to alter gene transcription or posttranscriptional events [69].

3.5. mediators and transmembrane signalling systems

The localization of the different receptors and their subtypes for inflammatory mediators in the pulmonary compartment and inflammatory cells has been reviewed in chapter one. In the present section, the formation of second messengers involved in the signal transduction of these mediators are discussed.

3.5.1. histamine

Occupancy of histaminergic subtype 1 (H_1)-receptors results in the stimulation of PI-turnover and the subsequent production of the second messengers IP_3 and DAG [25,50]. The increase in cGMP which occurs in the lung via H_1-receptor activation [97] is probably secondary to the increase in intracellular calcium, which occurs in response to PI-hydrolysis and IP_3 formation [8,86]. H_2-receptors are coupled to AC and occupancy of these receptors results in the production of cAMP [97]. H_3-receptors have been differentiated from H_1 and H_2-receptors using the selective agonist α-methyl histamine and the antagonist thioperamide, but the role of pulmonary H_3-receptors as well as the putative second messenger system involved in the signal transduction remain to be investigated [6].

3.5.2. prostaglandins

Prostacyclin (PGI_2) receptors have been identified in guinea pig lung by measuring the activation of AC and by direct receptor binding, probably localized to pulmonary vessels [54,78,79]. Thus, prostaglandins of the E-type and PGI_2 are known to stimulate AC whereas thromboxane A_2 is thought to inhibit AC via interaction with G_i [11,108]. Finally, $PGF_{2\alpha}$ exerts its effects through stimulation of PI-turnover [23,133].

3.5.3. leukotrienes

Using different celltypes (e.g. PMN's, smooth muscle cells and endothelial cells) the signal transduction of leukotrienes has been elucidated. Leukotriene B_4, LTC_4 as well as LTD_4 are coupled via G_i proteins to stimulate phospholipase C and PI-turnover to yield IP_3 and DAG [21]. The second messenger involved in the signal transduction of LTE_4 is still unknown. The HETE's (5HETE, 12HETE and 15HETE) have been shown to increase intracellular levels of cGMP [46].

3.5.4. platelet activating factor

The second messenger systems involved in the PAF-induced effects have been the subject of extensive studies. Depending on the cell type studied, PAF has been shown to both stimulate [48] and inhibit [59,138] AC, stimulate the PI-turnover and alter intracellular calcium levels [71,82,117] Still, no clear consensus has been reached for the exact mechanism involved in the transmembrane signal transduction of PAF. A possible explanation for this discrepancy could be the fact that PAF affects second messengers in an indirect way. In this respect, a close relationship between PAF and arachidonic acid metabolites has been implicated. PAF and arachidonic acid are not only released from a common precursor, 1-alkyl-2-acyl-glycero-3-phosphocholine [121] but it has also been shown that PAF induces the release of both cyclooxygenase and lipoxygenase metabolites [72,101,120]. As denoted above, cyclooxygenase and lipoxygenase metabolites have quite different effects on second messenger production. Therefore, depending on intracellular physiology (like $[Ca^{2+}]$), arachidonic acid is converted via cyclooxygenase or lipoxygenase into prostaglandins or leukotrienes which ultimately determine the formation of second messengers. In this respect, it is even tempting to speculate that PAF itself may act as a second messenger. Indeed, Stewart and Phillips [124] suggested a role for PAF as second messenger as agents which stimulate macrophages (like fMLP) also stimulate the generation of intracellular PAF and subsequent arachidonic acid mobilization.

3.5.5. neuropeptides

Information about transmembrane signalling systems of neuropeptides is limited. Pulmonary VIP-receptors (on bronchial smooth muscle and several inflammatory

cells) appear to be coupled through G proteins to the stimulatory site of AC [37,103]. Tachykinin receptors like substance P are probably coupled to PI-turnover [49].

3.5.6. complement

The second messenger(s) involved in the signal transduction of the complement factors are not known. Considering their biological activities (chapter one), PI-turnover is presumably involved.

3.5.7. cytokines

The signal transduction of IL-1 receptors involves a G protein [95] but it is still unclear which second messengers are associated with this G protein as conflicting results have been reported. Thus, IL-1 receptor occupancy does not affect or increases the concentrations of cAMP and DAG [1,17,88,116]. The second messengers involved in TNF- and IFN-induced signal transduction are not known.
The intracellular actions of growth factors like PDGF, CSF, MIP-1, TGF-ß and IGF-1 are most likely to be linked to activation of phospholipase C but the mechanisms involved are still poorly understood [90].

3.6. receptor desensitization

Prolonged exposure of the receptor to agonists results in progressive reduced effectiveness of the agonist. This refractoriness or desensitization is common to many receptor systems but has been extensively studied using the ß-adrenergic receptor transduction system as a model. Two types of desensitization have been defined: homologous and heterologous desensitization [127]. Homologous desensitization refers to the loss of responsiveness to the specific stimulating agent whereas the response to other agents activating the same effector system (AC in the case of ß-adrenergic agonists) remains unaffected. Conversely, heterologous desensitization occurs when exposure to one agonist results in the decreased response to multiple agonists operating via distinct receptors. Both types of desensitization are accompanied by phosphorylation of the receptor leading to a decreased response. However, heterologous desensitization appears to be mediated by the cAMP-dependent protein kinase A as cAMP analogues have been shown to induce this type of

desensitization [118], Furthermore, apart from the receptor, several studies indicated that other components of the AC signalling system (the G protein and the catalytic moiety) may be altered as well [116,119]. On the other hand, homologous desensitization (of the ß-receptor) is a cAMP-independent phenomenon and appears to be mediated by protein kinase called $ß_{ark}$ (ß-adrenergic receptor kinase) [125].
Since the original hypothesis by Szentivanyi [130], who postulated a general dysfunction in the ß-adrenergic transduction system in atopic diseases (including bronchial asthma), many investigators have tried to establish such a dysregulation of the ß-adrenergic signalling system with conflicting results [5,91]. It was concluded that alterations in ß-adrenergic function are not an intrinsic component of asthma but rather a consequence of the active disease. Indeed, ß-adrenoceptor dysfunction can be induced by several agents like viruses [15], oxygen radicals [28] and inflammatory mediators such as PAF [2]. Similarly, as ß-receptors are affected by exogenous factors, other receptors, like histaminergic and prostanoid, might have been altered in an analogous way. Thus, desensitization of receptors for histamine [14,83], PAF [45,94], and prostanoids [41,99] have also been reported. The mechanisms of desensitization of these receptors remain to be elucidated but, as the foregoing data suggest, it appears that several second messenger-stimulated protein kinases play a major role in this phenomenon.

3.7. regulation of activity of macrophages

The majority of studies determining the functional activity of macrophages in response to alterations in concentrations of second messengers have been carried out using rodent peritoneal macrophages. Limited data are available using alveolar macrophages (especially of human origin) considering second messenger production and functional activity. As table 3.2. shows, most macrophage functional features (phagocytosis, production of cytokines, lysosomal enzymes, cyclooxygenase metabolites and oxidative burst) are down-regulated by increased intracellular levels of cAMP whereas increased cGMP concentrations and PI-turnover generally result in up-regulation of these features. It should be noted however that, with regard to functional activity, second messengers may interact synergistically or have opposite effects. For instance, PAF stimulates the liberation of arachidonic acid (probably via enhanced PI-turnover) but this effect is counteracted by cAMP-increasing agents like ß-adrenergic agonists [3].

Table 3.2. The effects of increased concentrations of second messengers on macrophageal functions.

second messenger	feature and effect	references
cAMP	phagocytosis ↓	4,24,29,35,42,**110**,140
	oxidative burst ↓	20,85,**129**
	migration ↓	53
	enzyme secretion ↓	36,40,84,136
	growth/proliferation ↓	60
	aa-metabolism ↓	3,63
	cyclooxygenase activity ↓	27
	IL-1 production ↓	12
	TNF-production ↓	66,100
	tumoricidal activity ↓	112
	ADCC ↓	35
	opening Na^+ channels ↓	105
cGMP	phagocytosis ↑	35,62,76,141
	enzyme secretion ↑	107
	oxidative burst ↑	34
	aggregation ↑	100
	replication ↑	51
	ADCC ↑	35
	TNF-production ↑	109
IP_3 and DAG	oxidative burst ↑	20,129
	aa-metabolism ↑	3,63,137
	enzyme secretion ↑	16

References of studies using AM are depicted in **bold** letters, using human macrophages in underlined letters and studies using human AM are depicted in **bold and underlined** letters. *Abbreviations*: aa, arachidonic acid; ADCC, anibody dependent cellular cytotoxicity; IL-1, interleukin 1; TNF, tumor necrosis factor; ↑, increase; ↓, decrease.

3.8. concluding remarks

This chapter summarizes the mechanisms of converting multiple extracellular signals to intracellular production of second messengers and the subsequent regulation of cellular activity of the macrophage in general and the alveolar macrophage in particular. Most of the studies have considered functional activity of peritoneal macrophages. Although several differences have been reported between peritoneal and alveolar macrophages [115,131,134], probably due to anatomical location and heterogeneity, the basic mechanisms of transmembrane signalling systems in both cell types are most likely to involve similar characteristics.

As can be concluded from these *in vitro* studies, functional activity of macrophages can be generally up-regulated by agents which increase the concentration of cGMP or stimulate PI-turnover. Down-regulation is generally obtained through interactions of agents with the adenylyl cyclase transmembrane signalling system to stimulate intracellular cAMP concentrations. With the extrapolation of these data to an *in vivo* situation, one should keep in mind that the net outcome of functional activity of macrophages results from interactions from many different mediators with their receptors and the subsequent intracellular interactions of second messengers. For instance, histamine and PAF, which are both released from pulmonary cells during anaphylactic reactions, can act either synergistically or antagonistically as both mediators interact with different types of receptors coupled to different transmembrane signalling systems (see above). To what extent interactions between several second messengers ultimately determine functional macrophage activity remains to be elucidated.

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chapter four
aims of the study

From chapter one, it can be concluded that despite still poorly defined mechanisms, inflammation is clearly associated with the pathogenesis of both asthma and COPD. In the complex symphony of pulmonary processes, in which each pulmonary cell type plays its part, the alveolar macrophage (AM) may be considered as the director. Besides its typical features of phagocytosis and antigen presentation, the cell produces a variety of mediators which serve to communicate with other individual cellular components in pulmonary inflammation. These mediators are not only produced and secreted by AM but also determine their activity (chapter two). Upon interaction with specific surface-receptors, mediators and external factors (primary messengers) stimulate cellular effector enzymes which subsequently promote the production of second messengers which, via several mechanisms ultimately regulate functional cellular activity (chapter three).

In order to characterize functional mechanisms of action of AM in their regulatory task within pulmonary inflammation, three lines of research were designed:

1. characterization of the stimulatory site of the adenylyl cyclase (AC) membrane transduction signalling system of AM;
2. to study the mechanisms by which platelet activating factor (PAF) interacts with AM;
3. to study the effects of exogenous stimuli on the production of cAMP, cGMP and functional activity of AM.

ad 1. The characteristics of the stimulatory site of the adenylyl cyclase (AC) transmembrane signalling system of AM were studied using a guinea pig model for human asthma to obtain a controlled model to study the AC responsiveness under normal, sensitized and antigen challenged conditions (chapters five and six). Sensitization of guinea pigs with ovalbumin represents an immunologic state which shows many similarities with human allergic asthma. Upon exposure to ovalbumin, the immune system of the guinea pig becomes activated which renders the animal extremely susceptible to a second challenge. An intratracheal challenge (booster) of sensitized guinea pigs to the same antigen (now allergen) results in an immediate bronchoconstriction (in parallel with human asthmatics). AM from naive (non-

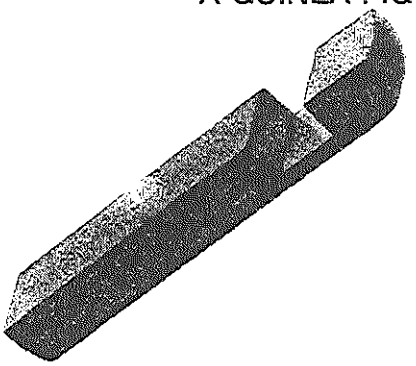
sensitized or controls), sensitized and antigen challenged animals (sensitized animals receiving a booster injection) were used to study the changes in stimulus-response coupling of the AC-system induced by (pre-) treatment. For this purpose the induction of cAMP production by several inflammatory mediators and ß-adrenergic agonists and its modulation by sensitization and/or antigen challenge were evaluated. Prostaglandin E_2 (PGE_2), prostacyclin (PGI_2 or DC-PGI_2, its stable analogue), and histamine were selected as inflammatory mediators (partly based on previous research and partly on their association with anaphylactic reactions) and isoprenaline and salbutamol as ß-adrenergic agonists which are frequently used as bronchodilators in the treatment of asthma and COPD. To elucidate which specific components of the AC signalling system would be involved in the modulation by sensitization and/or antigen challenge, the AC responsiveness was determined in membrane fractions of the different AM populations (chapter six).
In a parallel study using human AM, a comparison was made between basal cAMP levels and AC responsiveness in AM from controls, COPD patients and asthmatics (chapter nine) in order to validate the findings of the animal studies in clinical conditions.

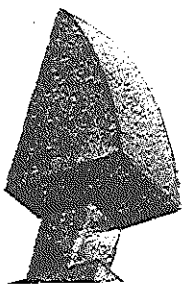
ad 2. The second part of the study was designed to elucidate the mechanisms by which PAF interacts with AM. PAF is a potent pro-inflammatory mediator in pulmonary diseases though its mechanism of action still is unclear. In this part of the study, we investigated whether PAF modulates the production and release of arachidonic acid metabolites and the production of cAMP in naive, sensitized and antigen challenged AM (chapters seven and eight).

ad 3. In the third part of the study, the effects of exogenous stimuli (smoking and drugs) on the production of cAMP, cGMP and the functional activity of AM were investigated. In chapter ten, the effects of ß-adrenergic and theophylline-like drugs on *in vitro* arachidonic acid metabolism of AM was studied and chapter eleven was disigned to investigate whether basal cAMP- and/or cGMP-levels correlate with spontaneous and the zymosan-induced release of arachidonic acid metabolites from AM obtained from smoking and nonsmoking controls and COPD patients.

PART TWO

A GUINEA PIG MODEL FOR ASTHMA





chapter five

antigen challenge modifies the cyclic AMP-response of inflammatory mediators and ß-adrenergic drugs in alveolar macrophages *

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5.1. summary

Adenylate cyclase activity was determined in alveolar macrophages obtained from BAL- fluids of naive (naive AMs) and antigen challenged guinea pigs. After the anaphylactic reaction in ovalbumin sensitized guinea pigs, basal levels of cyclic AMP in AMs were significantly increased compared to naive AMs (1.87 ± 0.22 versus. 5.26 ± 0.45 pmol cyclic AMP/5·10^6 cells). Prostaglandin E_2 (PGE_2), prostacyclin (DC-PGI_2), histamine, isoprenaline and salbutamol stimulated adenylate cyclase activity more effectively in AMs obtained from sensitized guinea pigs after the booster-injection compared to AMs obtained from non treated animals. Moreover, DC-PGI_2 and histamine which are hardly able to induce a rise in cyclic AMP levels in naive AMs, become effective activators in AMs obtained after antigen challenge (100 % and 60 % increase in response respectively). Using selective receptor ligands it is shown that $ß_2$- adrenoceptors and H_2- subtype histaminergic receptors are functionally coupled to macrophage adenylate cyclase activity. The present data indicate that sensitization does not effect the configuration of the receptor on the outer membrane (no change in affinity constants), but affects other parts of the transmembrane signal system leading to intracellular cyclic AMP production (e.g. regulatory binding proteins or increases in the number of receptors).

key words: alveolar macrophages, cyclic AMP, adenylate cyclase, inflammatory mediators, ß-adrenoceptor agonists,histamine

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5.2. introduction

Bronchial hyperresponsiviness and bronchial obstruction are common symptoms in patients with Chronic Obstructive Pulmonary Diseases (COPD), a collective name for several pulmonary disorders, including bronchial asthma, chronic bronchitis and emphysema [19]. The precise mechanism underlying these phenomena is largely unknown, but recent evidence suggests that airway inflammatory processes, induced by mediators released from various cells, is crucial in the pathophysiology of COPD [4,7].

Mast cells, eosinophils and epithelial cells make up 3- 5% of the cellular composition of bronchoalveolar lavage (BAL) fluids in normal subjects, whereas the majority (>90%) consists of alveolar macrophages (AMs). These cells form the first line of host defense against inhaled pathogens, mainly through phagocytosis but also through the production and release of lysosomal enzymes, proteases, and O_2^- metabolites [8]. Furthermore, AMs release inflammatory mediators such as arachidonic acid metabolites of the lipoxygenase and cyclooxygenase pathway (leukotrienes and prostanoids).

Many cellular functions of the macrophage are closely correlated to intracellular concentrations of cyclic AMP, as cell-mediated cytotoxicity, the release of lysosomal enzymes and phagocytosis are inhibited by increased intracellular cyclic AMP contents [6]. Rat peritoneal macrophages possess a feedback- mechanism in which newly synthesized prostaglandins (prostaglandin E2 and prostacyclin) inhibit, via cyclic AMP formation, their own production [15]. These results suggest that rises in intracellular cyclic AMP levels are reflected with reduction of cellular activity.

The role and action of histamine in COPD is clearly defined, though the distribution of airway histamine receptors in both human and animal models is somewhat puzzling. Bronchial smooth muscle cells probably possess H_1- subtype receptors coupled to guanylate cyclase. The presence of functional histaminergic receptors on alveolar macrophages remains however uncertain. Using monoclonal antibodies to cyclic GMP, Sertl *et al.* [26] described the presence of H_1- receptors on guinea pig AMs but smooth muscle cells and macrophages appear to lack H_2- histaminergic receptors [23,25].

Adenylate cyclase activity is not only affected by the action of inflammatory mediators, but is also regulated by neurotransmitters and hormones like adrenaline which react with adenylate cyclase through adrenergic receptors. ß- Adrenoceptor

agonists, drugs commonly used in the treatment of asthma, stimulate smooth muscle adenylate cyclase which results in increased cyclic AMP levels and bronchodilatation. The action of these drugs on macrophages however, is poorly documented. Verghese and Snyderman [28] described the stimulating effect of isoprenaline on adenylate cyclase in guinea pig peritoneal macrophage membranes, and Bachelet *et al.* [3] and Henricks *et al.* [11] reported recently a rise of intracellular levels of cyclic AMP in guinea pig AMs by ß- adrenoceptor agonists. The physiological implications of these events remain as yet unclear.
To establish a possible modulatory role for the alveolar macrophage in asthmatic conditions, we investigated the effects of the inflammatory mediators prostaglandin E2 (PGE_2), prostacyclin (DC-PGI_2), and histamine and the ß- adrenoceptor agonists isoprenaline and salbutamol on changes in intracellular cyclic AMP levels of alveolar macrophages of naive and antigen challenged guinea pigs (sensitized animals receiving a booster- injection). Furthermore, we present evidence for the existence of functional H_2- receptors on guinea pig AMs, coupled to adenylate cyclase.

5.3. methods

5.3.1. materials

Sodium pentobarbitone (Sodium pentobarbital) and cimetidine (free base) were obtained from the Pharmacy Department, Dijkzigt Hospital Rotterdam, The Netherlands. Prostaglandin E_2, (-) isoprenaline hydrochloride and salbutamol hemisulfate were purchased from Sigma Chemicals (St. Louis, MO, USA). IBMX (3-isobutyl-1-methyl-xanthine) was from Janssen Chimica (Beerse, Belgium), histamine hydrochloride from OPG (Utrecht, The Netherlands), mepyramine maleate from Rhône Poulenc (Paris, France) and atenolol (free base) and propranolol hydrochloride from ICI (Cheshire, UK). [^{3}H] cyclic AMP (specific activity : 36 Ci/mmol) was obtained from Amersham (Amersham, UK) and ovalbumine (chicken egg albumine) from Calbiochem (San Diego, USA). Ficoll Isopaque (Lymphoprep) was from Nycomed (Oslo, Norway). DC-PGI_2 ((±)-5E-13,14-didehydro-carbo-prostacyclin), a stable analogue of prostacyclin, was obtained through courtesy of Prof. C.A. Gandolfi, Farmitalia Carlo Erba (Milan, Italy).

5.3.2. sensitization

Male Hartley guinea pigs (weighing 300- 500 g) were sensitized by injecting ovalbumine, dissolved in 0.9 % saline, 50 mg subcutaneously and 50 mg intraperitoneally. Two weeks later, the animals were anaesthetized and bronchoalveolar lavage was performed.

5.3.3. antigen challenge and bronchoalveolar lavage

Animals were anaesthetized by injecting 70 mg kg^{-1} i.p. sodium pentobarbitone, the trachea was cannulated and bronchoalveolar cells were collected by repeated lavages of 8 ml aliquots of 0.9% saline (naive guinea pigs). Prior to bronchoalveolar lavage, lungs of sensitized animals were filled with 8 ml 0.9% saline containing 50 µg ovalbumin (booster- injection). After 45 seconds the lungs were normally lavaged with 0.9% saline (antigen challenged guinea pigs; modified from [20]). Besides naive guinea pigs (no treatment) and antigen challenged guinea pigs, one control group of animals was included in this study consisting of sensitized guinea pigs not receiving the booster- injection after two weeks. Bronchoalveolar cells were recovered from the lavage fluid by centrifugation (800 x g, 10 min at 4°C) resuspended in Gay Balanced Salt Solution (pH 7.4) and a Ficoll-Isopaque gradient centrifugation procedure was carried out (400 x g, 30 min at 4°C). Enriched macrophage populations were differentiated by May Grünwald Giemsa staining. Viabilities ranged from 90-95 % as tested by trypan blue exclusion.

5.3.4. experimental protocol

One ml samples containing 3.10^6 cells were incubated for 15 min at 37°C, in the presence of 400 µM IBMX (a phosphodiesterase inhibitor), with PGE_2, DC-PGI_2, histamine, isoprenaline or salbutamol. The latter three compounds were also tested in the presence of the antagonists mepyramine or cimetidine and atenolol or propranolol in final concentrations of 10 µM. After the incubationperiod cyclic AMP concentrations were determined by a high affinity protein binding method as was described earlier [5]. Cyclic AMP values were expressed as pmol/$5{\cdot}10^6$ cells.

5.3.5. statistical analysis

The data are expressed as means ± S.E.M. Statistical significance was evaluated by the unpaired Student's t-test.

5.4. results

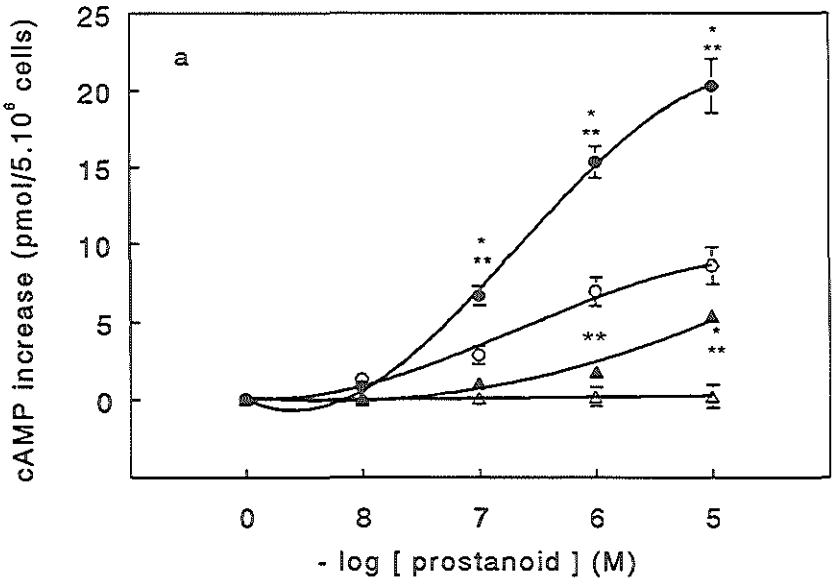
Table 5.1. shows that bronchoalveolar lavage fluids recovered from naive and antigen challenged animals did not differ in cellular composition as similar ratios were found of macrophages, lymphocytes, eosinophylic and neutrophylic granulocytes in both preparations using May Grünwald Giemsa staining. Total number of cells obtained through lavage, however, was markedly increased (43%) in antigen challenged animals as compared to naive animals.

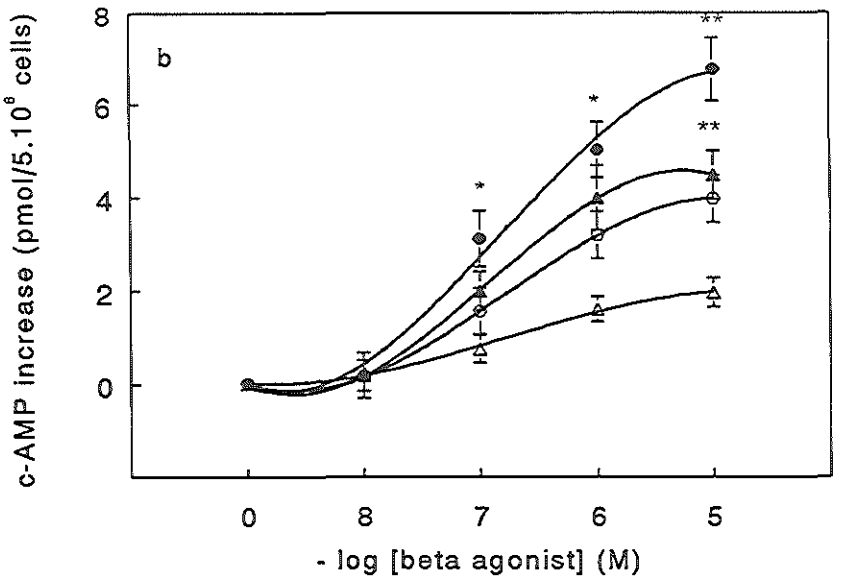
Table 5.1. Cellular composition and total cellcount of bronchoalveolar lavage fluids of naive and antigen challenged guinea pigs.

cellular composition (%)	naive (n=18)	antigen challenged (n=20)
macrophages	92.1 ± 0.41	91.8 ± 0.43
eosinophylic granulocytes	6.70 ± 0.45	5.80 ± 0.32
neutrophylic granulocytes	0.54 ± 0.08	0.90 ± 0.20
lymphocytes	0.57 ± 0.12	1.19 ± 0.28
mononuclear cells *	0.09 ± 0.05	0.32 ± 0.13
total cellnumber (x 10^6)	27.0 ± 0.78 (n=59)	38.7 ± 2.11 ** (n=27)

Number of observations in parenthesis. *: other than macrophages and lymphocytes; **: $p < 0.001$ compared to naive.

Antigen challenge in sensitized animals induces a pronounced rise in intracellular basal cyclic AMP levels of alveolar macrophage. Compared to naive AMs, basal cyclic AMP levels are elevated some 3 fold in antigen challenged animals (1.87 ± 0.22 (n=20) versus 5.26 ± 0.47 (n=16) pmol cAMP/5.10^6 cells; P<0.001). Basal cAMP levels in AMs obtained from sensitized animals (no booster-injection) were elevated by 86 % compared to naive AMs (3.48 ± 0.20 (n=20) versus 1.87 ± 0.22 (n=20) pmol cAMP/5.10^6 cells; P<0.01).


a
cAMP increase (pmol/5.10^6 cells)
25
20
15
10
5
0
0
8
7
6
5
- log [prostanoid] (M)


b
c-AMP increase (pmol/5.10^6 cells)
8
6
4
2
0
0
8
7
6
5
- log [beta agonist] (M)

5.1a/b

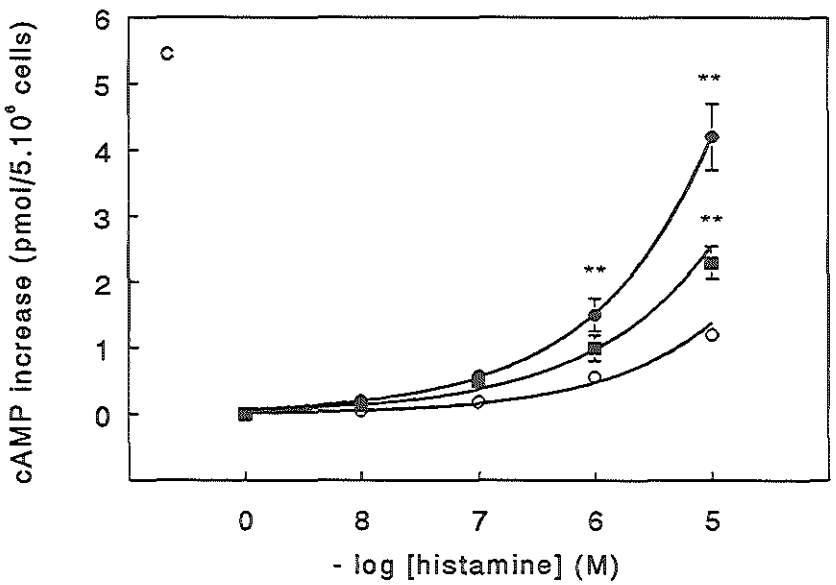


Figure 5.1. Effect of cyclic AMP increasing agents in AMs of naive (open symbols) and antigen challenged (closed symbols) guinea pigs. Responses are expressed as absolute increase in pmol cyclic AMP/5.10^6 cells. (a) PGE_2 (o) and DC-PGI_2 (Δ); (b) (-)isoprenaline (o) and salbutamol (Δ); (c) histamine, closed squares: control AMs (sensitized without booster-injection). Data are the means ± S.E.M. for 4-9 duplo experiments. $^*P<0.02$; $^{**}P<0.002$; $^{***}P<0.001$, compared to naive AMs. For details see Methods.

The effects of PGE_2, DC-PGI_2, histamine, and ß-adrenergic agonists on cyclic AMP levels in AMs obtained from naive and antigen challenged animals are shown in figure 5.1.. A large difference between AMs from naive and antigen challenged animals is observed regarding the activity of adenylate cyclase. versus 4.20 ± 0.68 $\cdot 10^{-7}$ M). Prostaglandin E_2, isoprenaline, salbutamol and histamine induced a dose-dependent increase of cyclic AMP levels in both naive and antigen challenged animals, while prostacyclin elevated cyclic AMP levels only in antigen challenged animals. Taking the absolute increase in cyclic AMP levels as a measure for adenylate cyclase activity, the efficiency of the drugs capable to increase cyclic AMP levels, is much higher in antigen challenged AMs than in naive AMs. The response of PGE_2 ($2.8 \cdot 10^{-8}$ M- $2.8 \cdot 10^{-5}$ M) on cyclic AMP levels was approx. 2 times larger in antigen challenged

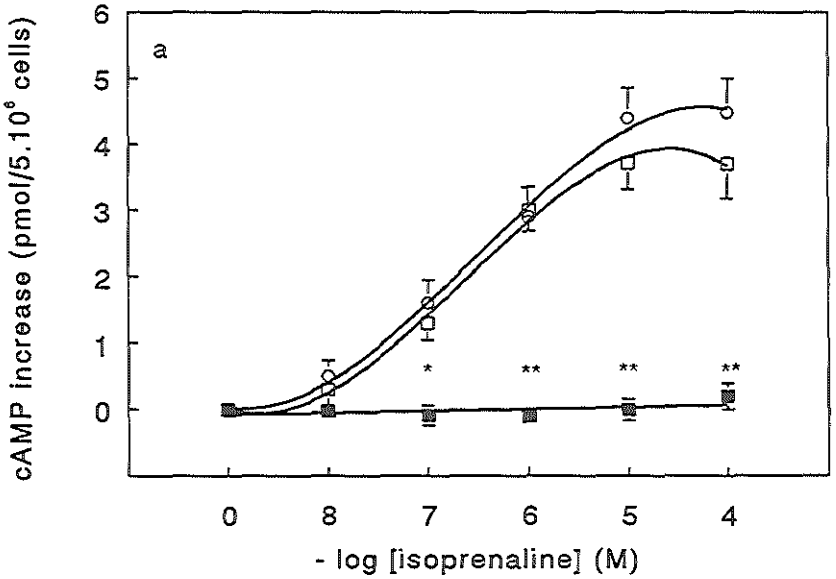
AMs compared to naive AMs though EC_{50} values remained the same (3.88 ± 0.88). However, DC-PGI_2 ($2.8 \cdot 10^{-8}$ -$2.8 \cdot 10^{-5}$ M) exerts a cyclic AMP elevating effect in antigen challenged animals, which was absent in the naive group (see fig. 1 panel A). Similar results were observed for the ß- adrenoceptor agonists and histamine (cf. fig.1 panel B and C respectively). Comparison of the effects on cyclic AMP levels by isoprenaline and salbutamol learns that the non- selective and full ß- adrenergic agonist isoprenaline induces a higher maximal absolute increase in cyclic AMP levels than the partial $ß_2$-selective agonist salbutamol (170% and 110% respectively). Both compounds have similar EC_{50} values when measured in the different AMs (2.66 ± 0.75 versus 1.83 ± 0.22 $\cdot 10^{-7}$ M and 4.37 ± 1.21 versus 5.05 ± 1.46 $\cdot 10^{-7}$M respectively). Apparently, the affinity of the ß- receptor for the ligands was not changed by sensitization.

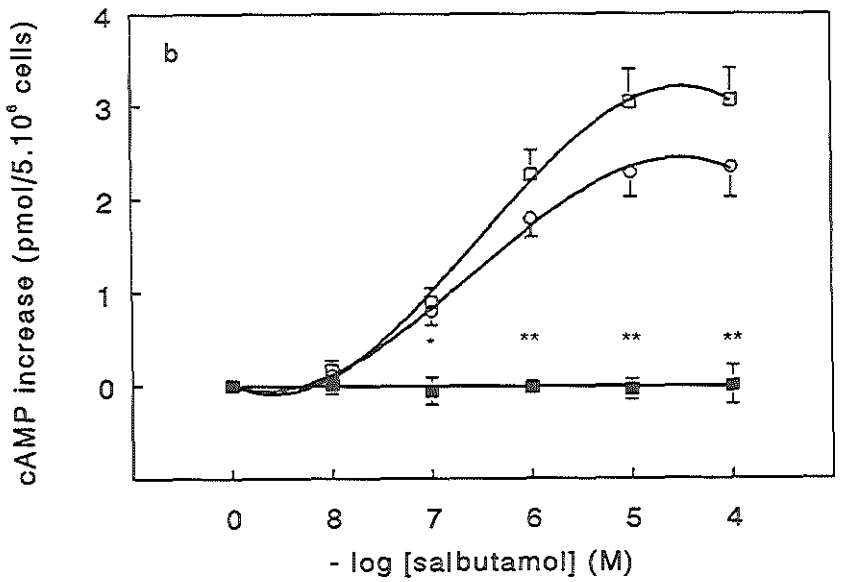
The largest difference between the adenylate cyclase activity in naive and antigen challenged AMs is observed when the cells are incubated with histamine (see fig. 1, panel C). At 10^{-6} M histamine becomes some 3-4 times more effective as cyclic AMP elevating agent in antigen challenged AMs compared to naive AMs. Furthermore, this panel also shows that the adenylate cyclase stimulatory activity in alveolar macrophages of sensitized guinea pigs (no booster- injection) due to histamine incubation is intermediate to the activity in naive and antigen challenged alveolar macrophages, as was already indicated by intermediate basal cAMP levels (1.87 < 3.48 < 5.26 pmol cAMP/ $5 \cdot 10^6$ cells).

Table 5.2. Effect of the different agonists on percentage increase of basal cyclic AMP levels of naive and antigen challenged (antigen) guinea pig AMs.

agonist	percentage increase			
	10^{-7}		10^{-5} M	
	naive	antigen	naive	antigen
PGE_2	75 ± 37.4	110 ± 22.3	440 ± 39.7	500 ± 56.0
PGI_2	12 ± 10.4	20 ± 9.3	25 ± 17.2	120 ± 22.2 *
histamine	10 ± 3.7	3 ± 4.6	65 ± 10.2	48 ± 16.4
isoprenaline	70 ± 17.7	115 ± 27.1	160 ± 20.7	175 ± 29.0
salbutamol	52 ± 12.9	70 ± 10.8	110 ± 18.6	120 ± 17.1

Data are expressed as means ± S.E.M. from 4-9 duplo experiments. *: $p < 0.001$ compared to naive AM.


a
cAMP increase (pmol/5.10⁶ cells)
6
5
4
3
2
1
0
*
**
**
**
0
8
7
6
5
4
- log [isoprenaline] (M)


b
cAMP increase (pmol/5.10⁶ cells)
4
3
2
1
0
*
**
**
**
0
8
7
6
5
4
- log [salbutamol] (M)

5.2.a/b

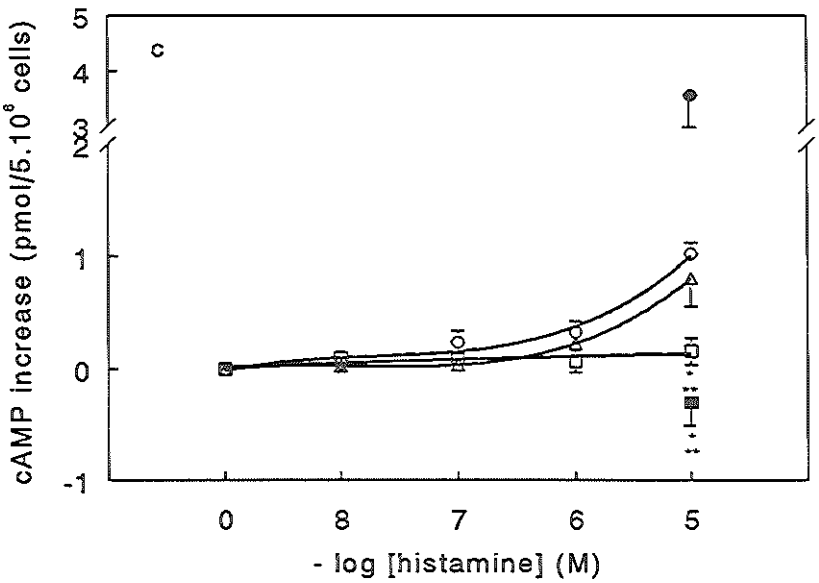


Figure 5.2. Effect of the ß- adrenergic antagonists atenolol (□) and propranolol (■) and the histamine antagonists mepyramine (Δ) and cimetidine (□) on the absolute increases of cyclic AMP levels of naive AMs induced by different ligands. (A) (-)isoprenaline; (B) salbutamol; (C) histamine, closed symbols: antigen challenged AMs, histamine 10 μM (●) and histamine 10 μM + cimetidine (■). Concentration of the antagonists was 10 μM. Data are the means ± S.E.M. from 2-3 duplo experiments. $^{*}P<0.02$; $^{**}P<0.01$; $^{***}P<0.001$, compared to values obtained in the absence of antagonists. For details see Methods.

In table 5.2. the absolute increases of basal cyclic AMP levels are represented in terms of percentage increase in cyclic AMP levels. These data show no differences in the cyclic AMP elevating effects of the inflammatory mediators PGE_2 and histamine when naive and antigen challenged AMs are compared. In both preparations the cyclic AMP levels were increased approx. 500 % by PGE_2 and 60 % by histamine. The same was valid for isoprenaline and salbutamol (170% and 110% respectively in both preparations). A large difference however, was found concerning the ability of PGI_2 to induce a rise of cyclic AMP levels in naive versus antigen challenged AMs. In naive AMs, prostacyclin ($2.8 \cdot 10^{-6}$- $2.8 \cdot 10^{-5}$ M) was not effective while in antigen challenged AMs, DC-PGI_2, in the same doses, increased cyclic AMP levels some

120%.
The effects of the ß-adrenoceptor antagonists atenolol ($ß_1$-selective) and propranolol (non-selective) on the cyclic AMP elevating effects of isoprenaline, salbutamol are depicted in figure 5.2.. Propranolol (10 µM) completely blocked the effects of both isoprenaline and salbutamol in naive guinea pig AMs (cf figure 5.2. panels A and B). In the same dose the $ß_1$- selective adrenoceptor antagonist atenolol could not reverse the effects of isoprenaline and salbutamol (cf figure 5.2., panel A and B).
In a similar way, to determine whether the histaminergic response of adenylate cyclase was mediated through H_1- or H_2- receptor subtypes, mepyramine (H_1-selective) and cimetidine (H_2- selective) were tested for antagonistic action on the histamine response. Figure 5.2. panel C shows that cimetidine (10 µM) but not mepyramine (10 µM) effectively blocked the cyclic AMP increasing effect of histamine in naive guinea pig AMs. Furthermore, cimetidine (10 µM) could also effectively block the much higher cAMP response of histamine (10 µM) which is observed in antigen challenged guinea pig alveolar macrophages.

5.5. discussion

In this report we describe the effects of inflammatory mediators and ß-adrenergic agonists on cyclic AMP production in guinea pig alveolar macrophages obtained through bronchoalveolar lavage of control and antigen challenged guinea pigs. Performing bronchoalveolar lavage immediately after antigen challenge (45 seconds), allowed us to investigate direct drug- induced changes in macrophage adenylate cyclase activity.
As 1) no qualitative differences in cellular composition of BAL fluids of naive and antigen challenged guinea pigs were found and 2) total cellnumber of BAL fluids in antigen challenged guinea pigs was increased, it can be concluded that the number of all inflammatory cells (macrophages, eosinophylic granulocytes and PMNs) is enhanced to the same extend by antigen challenge. As a result of antigen challenge, inflammatory mediators have been released into the airways which attract typical inflammatory cells such as eosinophils, macrophages and PMNs [4,7]. The increase in the number of eosinophils is in agreement with earlier findings in which an eosinophilic migration into the airways was observed by examining BAL fluids of patients with Late Asthmatic Response and of albumin sensitized guinea pigs during the late reaction induced by allergen challenge [18,13]. Similarly, the increase of

pulmonary macrophages is probably due to the migration of macrophages from the interstitium towards the alveoli and to an increase of macrophage precursors, the blood monocytes. Such an increase in the number of pulmonary macrophages is not found in human asthmatics, presumably because of the much longer duration of antigen exposure in asthmatic patients compared to sensitized animals (months/years versus days).

During inflammatory processes in the airways, arachidonic acid metabolites (leukotrienes and prostanoids) are released from mast cells and alveolar macrophages [21,24]. Subsequently, a rise of intracellular cyclic AMP levels of alveolar macrophages in antigen challenged animals can be expected, as arachidonic acid metabolites of the cyclooxygenase pathway are known to stimulate adenylate cyclase [1,2].

Likewise, histamine, released from mast cells during the anaphylactic shock, will interact with its receptors. In human antigen challenged lung-tissue, histamine induced a 3-fold increase in cyclic AMP levels via stimulation of H_2- receptors [22]. The present results extend these findings to alveolar macrophages. Apparently, alveolar macrophages possess functional H_2- receptors which are coupled to adenylate cyclase. This observation, together with the described ability of arachidonic acid metabolites, generated via the cyclooxygenase pathway, to stimulate adenylate cyclase, suggests that mediators, released during the anaphylactic reaction, may be responsible for the observed rise in basal cyclic AMP levels.

The use of selective agonists and antagonists generally allows to determine via which receptor subtype a certain pharmacological action is mediated. The results, using propranolol and atenolol as ß- adrenoceptor antagonists, strongly indicate the presence of only functional $ß_2$-adrenoceptors; propranolol (non-selective) completely blocked the effect of both isoprenaline and salbutamol whereas the $ß_1$- selective adrenergic antagonist atenolol showed no effect. These results are in agreement with previous experiments using guinea pig alveolar and peritoneal macrophages in which isoprenaline and salbutamol induced an increase in intracellular cyclic AMP levels [3,28]. Previous observations in human lymphocytes [16], a human monocytic-like cell line [10] and human polymorphonuclear neutrophils [9] showed that H2- receptors are coupled to the adenylate cyclase system. Our experiments extend these findings to alveolar macrophages. Using mepyramine and cimetidine as antagonists, we showed that the cyclic AMP- increasing effect of histamine in alveolar macrophages is mediated by H_2- receptor activation. Besides the known contraction by histamine of bronchial

smooth muscle mediated via H_1- receptor activation, we now show that histamine exerts another and novel effect in the pathogenesis of airway inflammatory diseases, i.e. the modulation of macrophage activity by increasing intracellular cyclic AMP levels via H_2- receptor activation.

Not only basal cyclic AMP levels but also the adenylate cyclase sensitivity was increased in AMs of antigen challenged guinea pigs. No distinction between naive and antigen challenged animals could however be made regarding the effects of the inflammatory mediators and ß- adrenoceptor agonists in terms of percentage increase of basal cyclic AMP levels (cf table 5.2.). This means that basal cyclic AMP level and the maximal attainable cyclic AMP level are closely correlated. In conclusion, the increase of basal cyclic AMP levels reflects well the prospective effect on the adenylate cyclase response.

This proposition does not apply to the effect of prostacyclin: this prostanoid fails to induce a response on cyclic AMP levels in naive AMs but increases macrophage adenylate cyclase activity in macrophages obtained from antigen challenged animals. Therefore these results can not be explained by the previous thesis, which included an unchanged adenylate cyclase complex. It is more likely that changes in its membrane transduction complex have occured during sensitization. Additional evidence for this hypothesis, which indicates that sensitization affects the adenylate cyclase complex comes from observations using AMs from the controlgroup: sensitization without a booster- injection induces basal cAMP levels and maximal increases of cAMP levels which are intermediate to naive and antigen challenged macrophages. Apparently, the observed changes in basal cAMP levels and adenylate cyclase sensitivity result from changes in this system which have occured during sensitization and from the interaction of during the booster- injection released mediators with the macrophage adenylate cyclase.

The observed rises in cAMP levels during antigen challenge seem to be in contradiction to an expected increase of macrophage activity as increases of intracellular cAMP levels are reflected with a decrease of cellular activity. There are two possible explanations for this phenomenon. Firstly, the interaction of stimulatory mediators which are released during antigen challenge with the adenylate cyclase system, which result in an increase of intracellular cAMP. Since we did not study the effect of antigen challenge over a longer period of time (i.e. longer than 45 seconds), we conclude that the initial effect of antigen challenge is a rise of basal cAMP levels in alveolar macrophages. Secondly, in this work we studied the effects of antigen

challenge on cAMP levels, which is only one of the known second messengers. Several cellular mechanisms of macrophages are closely correlated to intracellular cAMP concentrations. This however, does not rule out that other second messengers, besides cAMP are involved in the regulation of cellular activity.
Furthermore, increasing evidence has been raised for the existence of ß- adrenergic receptors on alveolar macrophages [11]. The physiological function of these receptors on alveolar macrophages remains however to be determined. It is generally accepted that ß- adrenergic receptors on bronchial smooth muscle cells are coupled to adenylate cyclase which upon stimulation result, via an increase of cAMP, in general bronchodilatation. For this reason these drugs are commonly used in the treatment of asthma, however increasing cAMP levels in alveolar macrophages is reflected with a down-regulation of several of the cell's important functions, e.g. phagocytosis. Therefore, the benificial effects of cAMP- increasing agents, like ß- adrenergic drugs and partly theophylline- derivatives, are as yet confined to their action on bronchial smooth muscle.
The adenylate cyclase system consists of three major components: receptors, the enzyme adenylate cyclase, and the connecting regulatory binding proteins. The affinity of PGE_2, isoprenaline and salbutamol for their respective receptors is not changed. It is therefore concluded that the conformation of the receptors is not altered by sensitization or the booster-injection. In addition, no change in the increase of cyclic AMP levels in terms of percentage increase indicates that the ability of adenylate cyclase enzyme itself to generate cyclic AMP is as well not affected by sensitization or booster-injection. This is in agreement with results from receptorbinding-studies in which no significant changes were found in the number and the affinity of ß- adrenergic receptors of sensitized guinea pig lungmembranes compared to controls [17]. Future experiments are necessary to determine whether changes on the level of the regulatory binding proteins or the receptor number are responsible for the described effects on intracellular cyclic AMP levels in alveolar macrophages.

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chapter six

sensitization enhances the adenylyl cyclase responsiveness in alveolar macrophages. changes induced at post-receptor level *

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6.1. summary

Using membrane fractions (MF) from guinea pig alveolar macrophages (AM), we investigated the effects of sensitization and antigen challenge on the stepwise activation of adenylyl cyclase (AC) considering receptorbinding, G-protein coupling and direct stimulation of the enzyme. Receptorbinding studies, using [^{125}I]-ICYP as ß-adrenoceptor specific ligand, show that neither receptor number (B_{max}) nor receptor affinity constants (K_d-values) were affected by sensitization or antigen challenge. Using forskolin as a direct stimulant of AC, alterations in the enzymatic activity of AC could be excluded. Pretreatment of the different MF with cholera toxin (CT, a toxin which eliminates GTP-ase activity) and subsequent stimulation of AC with GTP, shows an increased responsiveness in MF from sensitized and antigen challenged AM. In addition, pretreatment of MF from naive AM with increasing doses of CT results in a maximal AC response at the higher concentrations used (50-100 µg/ml), an effect not observed in MF from sensitized and antigen challenged AM. In these MF, the AC response still increases after pretreatment with such doses of CT. These data suggest that the enhanced AC responsiveness in AM, induced by sensitization and antigen challenge, results from alterations in α_s-subunits.

Key words: alveolar macrophages, adenylyl cyclase, cyclic AMP, cholera toxin, transmembrane signalling system, G protein, α_s-subunits

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6.2. introduction

Recently, substantial evidence has been raised supporting a role for alveolar macrophages (AM) in pulmonary inflammatory processes accompanying bronchial hyperreactivity in asthma. Upon exposure to several stimuli, AM from asthmatic patients, compared to AM from control subjects, show enhanced release of reactive oxygen species [7], lysosomal enzymes [22,17] and different inflammatory mediators like PAF-acether and leukotrienes and IL-1 [18,12,10].

Macrophages may respond to certain hormones and inflammatory mediators (like prostaglandins) by stimulation of adenylyl cyclase resulting in enhanced cAMP-levels which generally induce a down-regulation of cellular activity (e.g. phagocytosis, cytotoxicity, lysosomal enzyme secretion and O_2^--production, [2,3]).

Ovalbumin sensitization of guinea pigs is a commonly used animal model to study allergic bronchial asthma. We previously described [1] that AM obtained from sensitized and antigen challenged guinea pigs showed, compared to AM from naive guinea pigs, a marked enhanced adenylyl cyclase reponse to various stimuli like ß-adrenergic agonists, prostanoids and histamine. As yet, it is unclear which processes induced by ovalbumin sensitization are responsible for the observed enhanced adenylyl cyclase responsiveness. In studies of desensitization mechanisms of adenylyl cyclase, alterations in several components have been considered: receptor density and receptor configuration [11], receptor-G-protein coupling [20] or the modulation of α-subunit quantity [21]. Possibly, sensitization has dysregulated such phenomena in an analogous -though opposite to desensitization -way.

The signal transduction system leading to the formation of c-AMP is complex as it comprises several interactions of closely related components. As outlined by Gilman [9], receptor occupancy promotes the dissociation of the heterotrimeric G_s-complex yielding free α_s-subunits which become activated through replacement of bound GDP by GTP. In the GTP-activated state, the α_s-subunits interact with adenylyl cyclase resulting in the production of cAMP. Hydrolysis of bound GTP by a GTP-ase, intrinsic to the α_s-subunit terminates the activating signal. The α_s-subunit reassociates with the $\beta\gamma$ -subunit subsequently decreasing the adenylyl cyclase activity and the system is primed for another activation cycle. Changes in the separate steps of this activation cascade will thus result in modified c-AMP production and ultimately in altered cellular activity. Indeed, using lung homogenates from ovalbumine sensitized guinea pigs, Gadd and Bhoola [11] have reported an increase in adenylyl cyclase

activity whereas the responsiveness to activation by ß-adrenergic agonists was reduced. They proposed a selective uncoupling of stimulatory receptors to the guanine nucleotide regulatory protein. In contrast, using tracheal spirals and lung parenchymal strips, Burka and Saad [12] have shown that ovalbumin sensitization of guinea pigs did not induce any change in the adenylyl cyclase activity of the airway tissues.
To elucidate whether the enhanced adenylyl cyclase responsiveness in AM originates from alterations in this membrane signalling transduction system, we examined the stepwise activation of adenylyl cyclase in alveolar macrophages, considering receptorbinding, G-protein coupling and direct activation of the catalytic unit.

6.3. methods

6.3.1. reagents

IBMX (3-isobutyl-1-methyl-xanthine), a phosphodiesterase-inhibitor, was obtained from Janssen Chimica (Beersse, Belgium) and GTP (guanosine-5'-triphosphate disodium salt, GMP-PCP (guanylyl (ß- -methylene)-diphosphonate, tetralithium salt) and ATP from Boehringer (Mannheim, FRG). Prostaglandin E_2, (-)-isoprenaline hydrochloride and cholera toxin (CT) were purchased from Sigma (St. Louis, USA). CT was preactivated shortly before use by incubation at 37 °C for 20 min with 30 mM dithiothreitol in 0.1 M phosphate buffer (pH 7.4) to release an enzymatically active A_1 fragment [13].

6.3.2. animals, sensitization and antigen challenge

Male Hartley guinea pigs (weighing 300-500 g) were used throughout the study. Naive animals were anaesthetized by i.p. injection of 70 mg/kg sodium pentobarbitone, the trachea was cannulated and bronchoalveolar lavage was performed by repeated lavages of 8 ml volumes of 0.9 % saline (total of 150 ml). Sensitized and antigen challenged alveolar cells were obtained as described before [1]. Briefly, animals were sensitized by i.p. and s.c. injection of an ovalbumin-solution (each 50 mg in 0.9 % sterile saline). After a two weeks latent period, the animals were either subjected to the normal lavage procedure, rendering sensitized macrophages or received a booster-injection of ovalbumin after which the lavage was proceeded

as normal, rendering antigen challenged macrophages.

5.3.3. isolation of alveolar macrophages

Broncho alveolar lavage fluids were filtered through surgical gauze and centrifuged at 400 x g for 10 min at 4 °C. After resuspension of the cellpellet in Gey Balanced Salt Solution (GBSS) alveolar macrophages were purified by a Ficoll-Isopaque (Nycomed, Oslo, Norway) gradient centrifugation (400 x g, 30 min, 4°C). More than 95 % of the cell suspension obtained by this method consisted of macrophages as judged by May Grünwald Giemsa staining of cytofuge preparations. Viability was tested by Trypan Blue exclusion and always exceeded 95%. The isolated cells were washed thoroughly with GBSS and stored at -70° C.

6.3.4. adenylyl cyclase assay

Membrane fractions (MF) of alveolar macrophages were prepared by disrupting the macrophages in sucrose buffer (0.25 M sucrose, 50 mM Tris/HCl, 25 mM KCl and 5 mM $MgCl_2$, pH 7.4) with a Potter-Elvehjem homogenizer followed by centrifugation at 50,000 x g for 120 min. The resultant membrane pellet was washed twice in a Tris/HCl buffer (50 mM Tris/HCl, 5 mM $MgSO_4$, 2 mM EGTA and 0.4 mM IBMX) by centrifugation. Protein content was measured according to the method of Lowry *et al.* [15]. Membrane fractions were resuspended at a final protein concentration of 1 mg/ml in the Tris/HCl buffer (pH 7.4). Aliquots of 40 µl membrane suspensions were incubated for 15 min at 30 °C in 40 µl Tris/HCl-buffer containing 1.6 mM ATP and 0.8 mg/ml bovine serum albumin in the presence or absence of forskolin, GTP or GMP-PCP. Pretreatment of MF with CT was performed by incubation of 4 separate portions of MF (1 mg/ml each) with 0, 25, 50 or 100 µg/ml CT for 16 hrs at 4 °C. The incubation was continued as described above, without previous washing of the treated MF, in the presence of 10^{-4} M GTP. After incubation, samples were boiled for 3 min and centrifugated for 3 min at 12,000 x g. Content of cAMP was determined by RIA using a high affinity binding protein as previously described [4] (cAMP-levels are expressed as pmol/mg protein/min)

6.3.5. [^{125}I]-ICYP bindingassay

Bindingstudies were performed as described before [14]. Briefly, membrane suspensions (10 μg) were incubated with increasing concentrations (5-200 pM) of (-)-3-[^{125}I]iodocyanopindolol ([^{125}I]ICYP) [in the absence and presence of 0.1 μM timolol to define total and non-specific binding. At a concentration of 80 pM [^{125}I]ICYP, the specific binding was 60 % of total binding. The incubation was performed in a total volume of 200 μl in 50 mM Tris/HCl, containing 10 mM $MgCl_2$ (pH 7.4 at 37 °C). After one hour, samples were rinsed with 50 mM Tris/HCl, containing 10 mM $MgCl_2$ (pH 7.4 at 4 °C) and filtered through glass fiber filters. This procedure was repeated once, whereafter the filters were washed with cold buffer. Radioactivity was counted using a gamma-counter with an efficiency of 68%.

6.3.6. data analysis

All data are expressed as the means ± standard error of the mean (SEM). Statistical significance was evaluated by the unpaired Student's t-test.

6.4. results

Basal adenylyl cyclase activities of MF from naive and antigen challenged AM are shown in table 6.1.. In the absence of GTP virtually no cAMP was formed whereas in the presence of 10^{-4} M GTP basal cAMP-levels increased 2.6 and 5.6 times in MF from naive and antigen challenged AM respectively. Interestingly, prostaglandin E_2 (PGE_2) and isoprenaline (ISO) were remarkable more effective stimulants of cAMP-production in MF from antigen challenged AM compared to naive AM as indicated by the absolute increase (Stim.P). These results are in accordance with our previous findings in whole AM-cell preparations in which we observed a similar 2.5-fold difference in responsiveness between antigen challenged-and naive AM [1].
It was subsequently attempted to elucidate at which level the stimulatory signal transduction pathway of adenylyl cyclase was modified by sensitization or antigen challenge. Thus, receptorbinding, G-protein coupling and enzymatic activity of the catalytic unit were considered.
Receptor bindingstudies were performed using the nonselective ß-adrenoceptor antagonist (-)-3-[^{125}I] iodocyanopindolol ([^{125}I]ICYP). Analysis of the binding data by

nonlinear regression revealed the presence of a single binding site for [^{125}I]ICYP in all three membrane fractions (naive, sensitized and antigen challenged). As shown before [18], this bindingsite was characteristic for β_2-adrenoceptors. As table 6.2. shows, equilibrium dissociation constants (K_d-values) were not altered by sensitization or antigen challenge. In addition, no significant differences were found regarding maximal binding (B_{max}, reflecting total receptornumber per mg protein) of the radioactive ligand to membranes of naive, sensitized and antigen challenged AM. Apparently, no changes in the conformation or the number of stimulatory ß-adrenergic receptors have been induced by sensitization or antigen challenge.

Table 6.1. Basal cAMP-levels and adenylyl cyclase response to PGE_2 and isoprenaline (ISO) in membrane fractions from naive, sensitized and antigen challenged alveolar macrophages.

agents	adenylyl cyclase activity (pmol cAMP/mg protein/min)					
	naive			antigen challenged		
	abs. value	(%)	Stim.P	abs. value	(%)	Stim.P
-GTP	5.6 ± 0.5	-	-	6.2 ± 0.5	-	-
+ GTP (10^{-4} M)	14.7 ± 1.5	-	-	34.2 ± 4.9*	-	-
GTP + PGE_2 10^{-8} M	15.5 ± 3.0	(5)	0.8	33.3 ± 2.3*	(0)	-0.7
GTP + PGE_2 10^{-6} M	18.8 ± 3.0	(28)	4.1	42.6 ± 4.7*	(25)	8.4
GTP + PGE_2 10^{-4} M	21.0 ± 3.3	(43)	6.3	67.3 ± 7.6**	(97)	33.1
GTP + ISO 10^{-8} M	19.3 ± 3.5	(31)	4.6	41.4 ± 5.9*	(21)	7.2
GTP + ISO 10^{-6} M	24.4 ± 6.6	(66)	9.7	59.2 ± 6.5*	(73)	25.0
GTP + ISO 10^{-4} M	27.5 ± 6.8	(87)	12.8	68.8 ± 5.8*	(101)	34.6

In brackets the % increase compared to basal cAMP-levels in the presence of 10^{-4} M GTP is given. Stim.P denotes cAMP-production (pmol/mg protein/min) over basal levels induced by stimulation of adenylyl cyclase with agonist. When PGE_2 and Iso were used, 10^{-4} M GTP (GTP) was added to the suspensions. Data are obtained from 22-30 duplo experiments (without receptor stimulus), from 5-6 duplo experiments (PGE_2 and ISO) and are expressed in pmol/mg protein/min and as means ± SEM. *$P<0.005$; ** $P<0.01$, as compared to the effect of the corresponding concentrations in naive membrane fractions.

In order to determine whether sensitization or antigen challenge affects the enzymatic activity of the catalytic unit, the effect of direct activation of adenylyl cyclase by forskolin was determined in MF from naive and antigen challenged AM. As depicted in figure 6.1., forskolin induces the same stimulatory effect on adenylyl cyclase in

both membrane preparations indicating no alteration in the catalytic properties of the enzyme by sensitization or antigen challenge.
Finally, we determined the effect of sensitization and antigen challenge on the

Table 6.2. Receptor density (B_{max}) and equilibrium dissociation constants (kd) of membrane fractions from naive, sensitized and antigen challenged alveolar macrophages.

	B_{max} (fmoles/mg protein)	K_d (pM)
naive	131 ± 14	79 ± 8
sensitized	127 ± 5	67 ± 5
antigen challenged	152 ± 29	62 ± 5

[^{125}I]-ICYP was used as nonselective ß-adrenoceptor ligand. Experimental procedure as described in Materials and Methods. Data shown are obtained from 3 duplicate experiments and are expressed as means ± SEM.

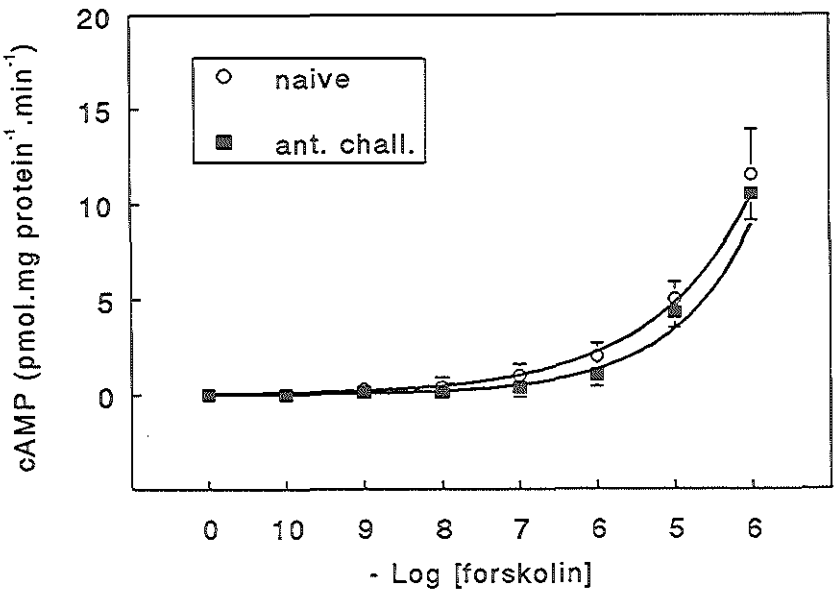

Figure 6.1. Effect of forskolin on adenylyl cyclase activity of membrane fractions from naive (o) and antigen challenged (▪) AM (in the absence of GTP). Incubation procedure as described in Materials and Methods. Data are expressed as mean absolute increases in pmol/mg protein/min of cAMP-levels ± SEM of 9 duplicate experiments.

transduction process regulated by G_s -proteins. Exposure of the MF to GTP enhances

adenylyl cyclase activity and the subsequent hydrolysis of GTP to GDP by GTP-ase activity intrinsic to the α_s-subunit, terminates this signal. Hence, it seemed that the enhanced basal adenylyl cyclase activity in sensitized and antigen challenged AM (cf. table 6.1.) could be due to a decreased GTP-ase activity of the α_s-subunit prolonging adenylyl cyclase activation. Since GTP is also able to stimulate G_i, an enhanced GTP-ase on the G_i-proteins in MF from antigen challenged AM would also explain the effects of GTP on basal cAMP-levels. Both theories imply that GTP-analogues, not susceptible to hydrolysis by GTP-ase, would show, in contrast to GTP, in all three MF a similar stimulatory response.

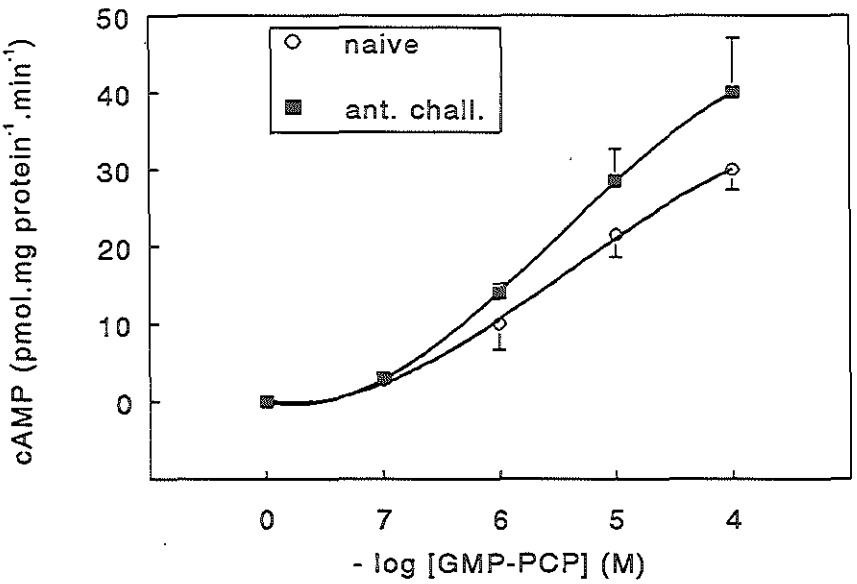

Figure 6.2. Effect of GMP-PCP and on adenylyl cyclase activity of membrane fractions from naive (o) and antigen challenged (▪) AM. Incubation procedure as described in Materials and Methods. Data are expressed as mean absolute increases in pmol/mg protein/min of cAMP-levels ± SEM of 9 duplicate experiments.

However, figure 6.2. shows, that the non-hydrolyzable GTP-analogue GMP-PCP, stimulates adenylyl cyclase slightly more effective in MF from antigen challenged AM as compared to naive MF though the difference is not significant. This minor effect is reflected by a divergement of the two dose-response curves which tend to result in different maximal values for adenylyl cyclase stimulation at higher (> 10^{-4} M) GMP-

PCP concentrations. For practical reasons (solubility) it was not possible to establish a significant difference in maximal effect.

The GTP-hydrolyzing enzymes residing on the α-subunit of G_s-proteins can be specifically inactivated with cholera toxin (CT) which will likewise result in a prolonged activation of adenylyl cyclase due to the accumulation of α_s in a GTP-liganded state. Pretreatment of MF of AM with increasing dose of CT and the subsequent challenge of adenylyl cyclase with a standard (maximal) dose of 10^{-4} M GTP thus provides other means to determine differences in GTP-ase activities among the different MF.

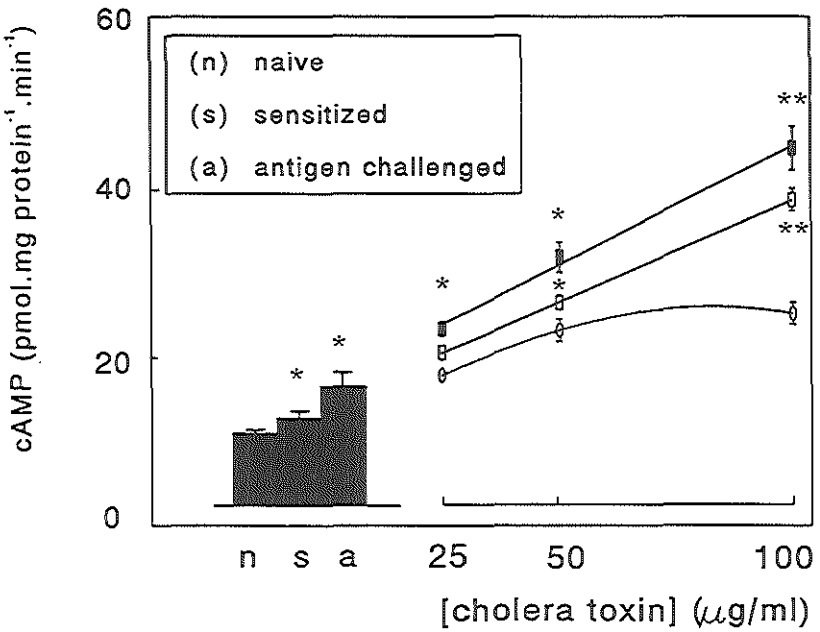

Figure 6.3. Effect of cholera toxin pretreatment of membrane fractions on the effect of 10^{-4} M GTP on adenylyl cyclase activity of membrane fractions from naive (o), sensitized (□) and antigen challenged (■) AM. Inset: "n", "s" and "a" refer to basal cAMP-levels in MF from naive, sensitized and antigen challenged AM respectively. Incubation procedure as described in Materials and Methods. Data are expressed as mean cAMP-levels (pmol/mg protein/min) ± SEM of 5 (naive), 3 (sensitized) and 5 (antigen challenged) duplicate experiments. * $P < 0.05$; ** $P < 0.005$ as compared to naive MF.

Figure 6.3. shows that the stimulatory activity of adenylyl cyclase in naive MF by 10^{-4}

M GTP clearly depends on the dose of CT used in the pretreatment period, as at low doses CT (< 50 μg/ml) GTP is still inactivated by some GTP-ase activity. Beyond 50 μg/ml of CT, a maximum is reached in the capability of 10^{-4} M GTP to stimulate adenylyl cyclase, suggesting complete elimination of GTP-ase activity after pretreatment of MF with CT at doses ≥ 50 μg/ml. However, in CT-pretreated MF from sensitized and antigen challenged AM, such maximal stimulation by 10^{-4} M GTP is not attained. Interestingly, 10^{-4} M GTP challenge of these pretreated MF results in cAMP-levels exceeding the maximal level observed in CT-pretreated naive MF. Secondly, irrespective of the doses of CT used in the preteatment period, 10^{-4} M GTP stimulates adenylyl cyclase in MF from antigen challenged AM more effectively as compared to naive AM. Under the same conditions the stimulatory action of 10^{-4} M GTP in MF from sensitized AM is comparable to the results obtained in MF from antigen challenged and corresponds with intermediate values.

6.5. discussion

Using ovalbumin sensitization of guinea pigs, we previously observed an enhanced adenylyl cyclase responsiveness in alveolar macrophages (AM) to different stimuli like ß-adrenergic agonists and inflammatory mediators [1]. We presently confirm this using membrane fractions (MF) of macrophages from naive, sensitized and antigen challenged guinea pigs. Regarding the difference in basal cAMP-levels in the presence of 10^{-4} M GTP (cf. Table 6.1.) a dysbalance in G_s/G_i-status in favour of G_s-stimulation has apparently been induced by antigen challenge. Whether G_i-pathways are impaired or G_s-pathways are enhanced cannot be determined merely on the basis of these data on basal cAMP-levels.
If impairment of G_i-pathways would have been responsible for the difference of 19.5 pmol/mg protein/min in basal cAMP-levels (in the presence of 10^{-4} M GTP), one should -upon stimulation with G_s-activating agents like isoprenaline and PGE_2 - observe the same difference in their response when MF from antigen challenged and naive AM are compared. This is however not observed. Using these two agonists, the differences exceed the value of 19.5 pmol/mg protein/min from which can be concluded that the extra stimulatory capacity of isoprenaline and PGE_2 in MF from antigen challenged AM is due to enhanced G_s-coupled receptor signal transduction. Sensitization and antigen challenge do not affect the configuration or number of ß-adrenoceptors (no change in K_d and B_{max} respectively) and therefore exclude the

possibility that such changes are, in addition, responsible for the enhanced adenylyl cyclase response in sensitized and antigen challenged AM.
No differences between the various MF are observed, considering direct stimulation of adenylyl cyclase by forskolin. Therefore, sensitization and/or antigen challenge apparently have not altered the catalytic site of the enzyme itself. Similar results were obtained by others [5,16] who showed that adenylyl cyclase activity of guinea pig lung smooth muscle homogenates was unaffected after a similar ovalbumine sensitization procedure.
Gadd and Bhoola [11] have recently suggested a reduction in GTP-ase activity of the α_s-subunits to explain a more effective adenylyl cyclase activation in guinea pig lung homogenates. GTP-ase promotes the hydrolysis of GTP to GDP which results in the de-activation of the GTP-liganded α-subunit and ultimately in the termination of the transduction signal. Differences in basal cAMP-levels determined in the presence of GTP (cf. table 6.1.) might well be ascribed to a decrease in GTP-ase activity on α_s or an increase in its activity on α_i in MF from antigen challenged AM. Such interference of GTP-ase activity can be determined using a non-hydrolysable GTP-analogue to promote the irreversible dissociation of the α-subunit from the $\beta\gamma$-subunit. Only minor differences in the adenylyl cyclase stimulatory response to GMP-PCP were observed comparing MF from naive and antigen challenged AM. Therefore, these results do not allow to firmly establish whether sensitization and/or antigen challenge has altered GTP-ase activity in either α_i-or α_s-subunits.
In analogy with studies which showed an increase in the number of α_i-subunits by desensitization [10], we considered whether sensitization would have induced an increase in the number of α_s-subunits. Such an increase would not only explain the enhanced basal cAMP-levels in MF from sensitized and antigen challenged AM, but also the supposed difference in maximal values of adenylyl cyclase stimulation by GMP-PCP. To elucidate the mechanisms of α_s-subunit activation and the subsequent coupling to adenylyl cyclase in further detail, we studied in the different MF (naive, sensitized and antigen challenged) the effect of pretreatment with cholera toxin on the GTP-induced adenylyl cyclase response. This toxin ADP-ribosylates specific amino acids of the α_s-subunits resulting in the inhibition of α_s-GTP-ase activity and constitutive activation of adenylyl cyclase due to the accumulation of α_s in a GTP-liganded state [10]. CT-pretreatment of MF and subsequent challenge with GTP thus enables to elucidate whether sensitization or antigen challenge reduce the GTP-ase activity. Obviously, at a CT concentration sufficient to completely eliminate GTP-ase

activity in naive MF, where the highest GTP-ase activity is retained, a similar stimulatory effect of 10^{-4} M GTP would be obtained in all three populations of MF. Moreover, the absolute value of the maxima, in terms of cAMP-production, would for all preparations be the same. The data however, show that pretreatment with CT in a dose of 50 μg/ml indeed appears to eliminate GTP-ase activity in naive MF but not in MF from sensitized and antigen challenged AM. Pretreating these MF with this dose of CT would, as delineated above, result in the same or even decreased stimulatory effect of 10^{-4} M GTP. Remarkably, such CT-pretreatment of MF from sensitized and antigen challenged AM results in cAMP-values exceeding the maximal value obtained with MF from naive AM. This effect is even more pronounced when MF are pretreated with a two-fold higher doses of CT (100 μg/ml). Apparently, pretreatment of MF from sensitized and antigen challenged AM with 50 μg/ml of CT is not sufficient to completely eliminate GTP-ase activity. It should be emphasized that elimination of GTP-ase activity would not result in an enhancement of the maximal stimulatory effect of GTP, but merely to a leftward shift of the GTP-dose response curve. Assuming an increment in the number of α_s-subunits by sensitization or antigen challenge could well explain the observed increase in maximal stimulation. Consequently, as such an increase in the number of α_s-subunits coincides with an increase in GTP-ase activity (residing on these subunits), more CT is necessary to fully inactivate the (enhanced) GTP-ase activity in antigen challenged compared to naive MF (cf. figure 6.3.). Despite the simultaneous increase in both α_s-subunits and GTP-ase activity by antigen challenge, an improved signal transduction is the net result suggesting that the enhanced GTP-ase activity is of less importance for the activation of adenylyl cyclase. Using other approaches like immunoblotting will enable to establish to what extend the number of α_s-subunits is affected by sensitization and/or antigen challenge.

As yet, we conclude that the enhanced responsiveness of adenylyl cyclase observed in sensitized and antigen challenged AM results from alterations in α_s-subunits of the signal transduction pathway induced by sensitization, ultimately leading to an enhanced cAMP-production and a subsequent decrease in cellular activity.

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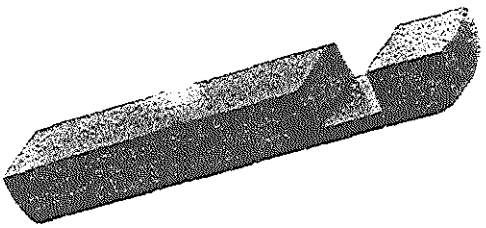
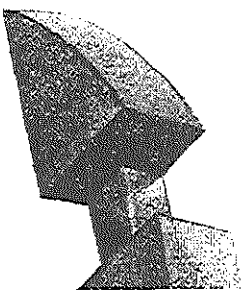
PART THREE

PLATELET ACTIVATING FACTOR AND ALVEOLAR MACROPHAGES





chapter seven

involvement of eicosanoids in platelet activating factor-induced modulation of adenylyl cyclase in alveolar macrophages *

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7.1. summary

Platelet activating factor (PAF) induces a dose dependent biphasic response on adenylyl cyclase activity in antigen challenged alveolar macrophages (AM), but not in naive AM. Intracellular cyclic AMP- levels are enhanced by very low concentrations of PAF (10^{-13}-10^{-10} M) and decreased by higher PAF- concentrations (10^{-8}-10^{-5} M). The PAF-effect on adenylyl cyclase could be completely blocked by pretreatment with the PAF- receptor antagonist BN 52021. The adenylyl cyclase stimulatory and inhibitory phase is reversed by indomethacin (inhibiting cyclooxygenase) and AA 861 (inhibiting lipoxygenase) respectively. These results show that the PAF-induced response on adenylyl cyclase activity in antigen challenged AM is achieved by its modulation of intracellular arachidonic acid metabolism.

Key words: Platelet Activating Factor, eicosanoids, cyclic AMP, alveolar macrophages, bronchial anaphylaxis, adenylyl cyclase, arachidonic acid metabolism

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* F.D. Beusenberg, J.M.E. van Schaik, J.G.C. van Amsterdam, I.L. Bonta. Involvement of eicosanoids in platelet activating factor-induced modulation of adenylyl cyclase in alveolar macrophages. J Lipid Mediators 1991;3:301-310. Printed with permission from the publisher.

7.2. introduction

Platelet Activating Factor (PAF), a lipid derived substance produced by macrophages, eosinophils, platelets and neutrophils [7], is an extremely potent mediator in bronchial hyperresponsiviness [3,18,22]. At the start of the bronchial anaphylactic reaction, PAF is momentarily released [9]. In this moment, alveolar macrophages form a major target cell for this pro- inflammatory mediator resulting in triggering of the immune system and the recruitment and activation of other cells (lymphocytes, eosinophils and neutrophils), known to be involved in the subsequent cascade of bronchial hyperreactive reactions [20].
Upon PAF- receptor stimulation, several second messengers may be produced, depending on the cell type studied. Both adenylyl cyclase stimulatory [10] and inhibitory [11,24] effects of PAF have been reported. In addition, PAF has been shown to enhance phosphoinositol (PI)- turnover [16,17,21], resulting in the generation of inositol tri phosphate (IP_3) and diacylglycerol (DAG). Secondly, upon stimulation of the PI-cycle, arachidonic acid may be released in a Ca^{2+}-dependent way [15]. Indeed, PAF has been shown to increase the liberation of arachidonic acid from cellular phospholipid pools and subsequently to stimulate eicosanoid release [2,5,8].
In human peritoneal macrophages we showed before that PAF stimulated adenylyl cyclase, which could be reversed by pretreatment with the PAF-receptor antagonist BN 52021 or the cyclooxygenase inhibitor indomethacine [1].
In guinea pig alveolar macrophages PAF increases the liberation of arachidonic acid which is counteracted by compounds which augment intracellular cyclic AMP- levels like Prostaglandin E_2 and ß- sympathicomimetics [2].
As both PAF and arachidonic acid metabolites are involved in anaphylactic bronchial reactions, we investigated the interrelationship between these mediators in alveolar macrophages (AM) with emphasis on PAF- induced modulation of adenylyl cyclase activity. Secondly, as we previously showed that sensitization and antigen challenge affects the adenylyl cyclase transmembrane signalling system [4], effects of PAF on cyclic AMP- production was determined in naive, sensitized and antigen challenged alveolar macrophages.

7.3. methods

7.3.1. animals and sensitization

Male Hartley guinea pigs (weighing 300 -500 g) were used throughout the study. Naive animals were anaesthetized by i.p. injection of sodiumpentobarbitone, the trachea was cannulated and bronchoalveolar lavage was performed by repeated lavages of 8 ml volumes of 0.9 % saline (total 150 ml).
To obtain sensitized and antigen challenged alveolar macrophages, animals were activily sensitized with ovalbumine as previously described [4]. Briefly, animals were sensitized by i.p. and s.c. injection of an ovalbumine solution (each 50 mg in 0.9 % sterile saline). Following a two weeks latent period, the animals were either subjected to the normal lavage- procedure (sensitized animals) or received a booster injection of ovalbumine after which the lavage was proceeded as normal (antigen challenged animals).

7.3.2. isolation and preparation of alveolar macrophages

The lavage fluids were filtered through surgical gauze and centrifuged at 400 x g for 10 min at 4° C. Resuspension of the cellpellets in Gay Balanced Salt Solution (pH 7.4) was followed by a Ficoll- Isopaque (Nycomed, Oslo, Norway)- gradient centrifugation (400 x g, 30 min, 4° C). After repeated washings, cells were resuspended in GBSS at concentrations of $3 \cdot 10^6$/ml. Cytofuge preparations were stained with May Grünwald Giemsa- staining. Viability of the cellsuspensions was tested by Trypan Blue exclusion and always exceeded 95 %.

7.3.3. incubation protocol

Samples containing $3 \cdot 10^6$ cells were incubated for 15 min at 37° C in the presence of 400 µM IBMX (3-isobutyl-1-methyl-xanthine, Janssen Chimica, Beerse, Belgium) and PAF (Sigma Chemicals, St. Louis, USA). When used, the antagonist BN 52021 (Ginkgolide B, a generous gift from Dr. P. Braquet, Le Plessis, Robinson, France) and inhibitors indomethacin and AA 861 (a 5-lipoxygenase inhibitor, kindly provided by Dr. S. Terao, Takeda Chemical Research Division, Osaka, Japan) were added to the cell suspensions on ice, 5 min prior to the incubation with PAF. After the incubationperiod, samples were boiled for 3 min. and cyclic AMP concentrations were determined using a high affinity protein binding method as described earlier [6].

7.3.4. data analysis

All data are expressed as the means ± standard error of the mean (SEM) . Statistical significance was evaluated by the unpaired Student's t-test.

7.4. results

As reported earlier, cytologic evaluation of the BAL fluids only showed differences in the total number of cells. The number of AM was increased by about 40% in both sensitized and antigen-challenged animals [4].

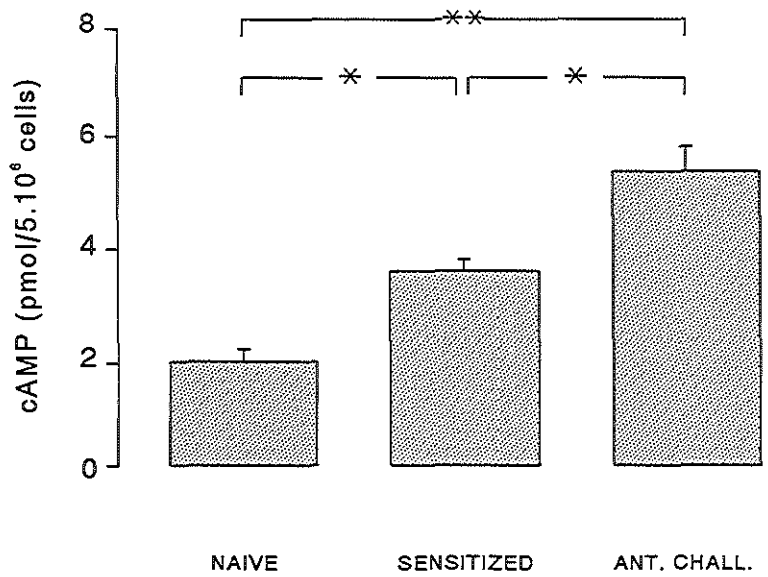
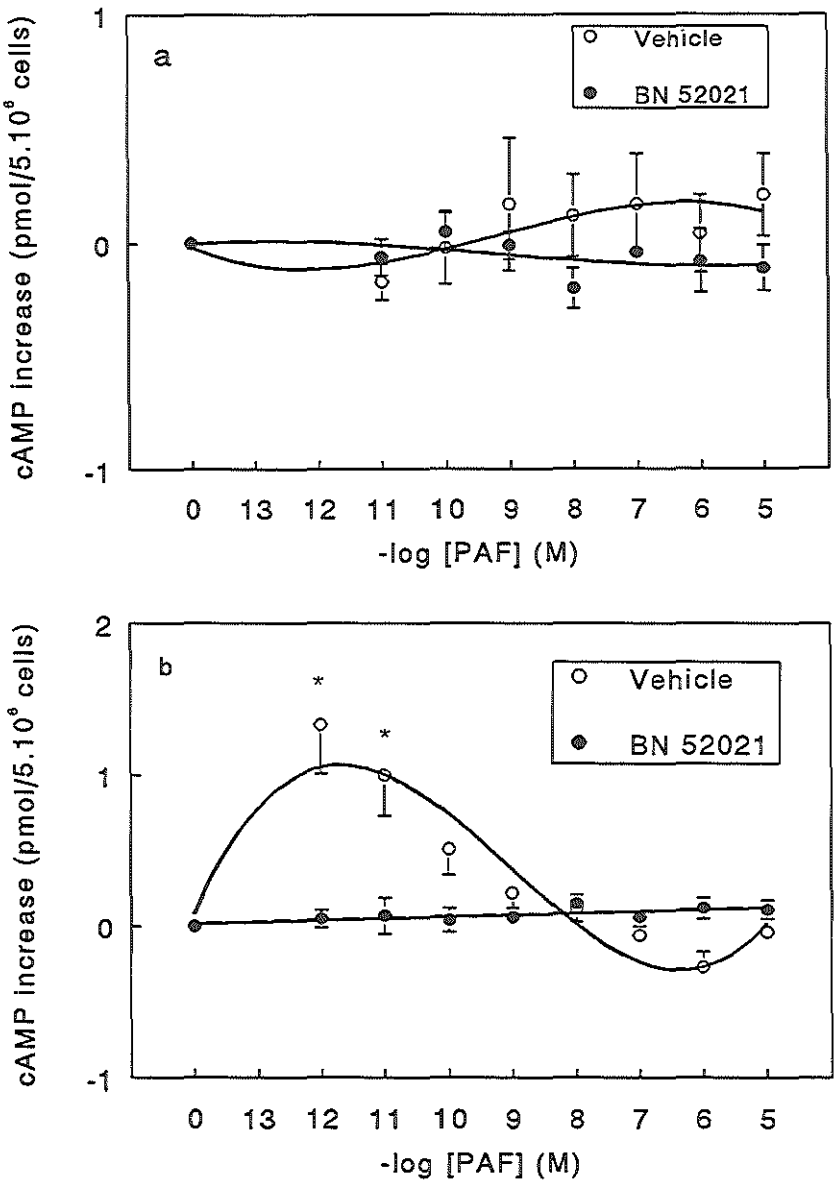


Figure 7.1. Basal cyclic AMP levels in naive, sensitized and antigen challenged (ant. chall.) AM. Data are obtained from 20, 20 and 16 duplicate experiments respectively and are expressed as pmoles c-AMP/5·10^6 cells ± SEM. Statistical significance was assessed by the Student's t-test (one tailed). *P<0.0005; **P<0.001.

As shown in figure 7.1., basal cyclic AMP levels were increased by 86% and 181% in sensitized and antigen-challenged AM respectively, compared to naive AM. Bearing this in mind, the effects of PAF on the cyclic AMP- response are represented as absolute increases in cyclic AMP- levels.



7.1 a/b

PAF induces a biphasic response on cyclic AMP- levels in AM of both sensitized AM (Figure 7.2.b) and antigen challenged AM (Figure 7.2.c) but did not affect cyclic AMP-

levels in naive AM (Figure 7.2.a).
Low concentrations of PAF (10^{-13}-10^{-10} M) increase cyclic AMP- levels whereas higher PAF- concentrations (10^{-8}-10^{-5} M) decrease intracellular cyclic AMP- levels. The latter effect was most pronounced in antigen challenged AM while it was not significant in sensitized AM (cf Figure 7.2.).
In addition, both the increasing and the decreasing effect of PAF on cyclic AMP-levels could be effectively blocked by the specific PAF- receptor antagonist BN 52021 (10 μM), indicating a receptor- mediated mechanism (Figure 7.2.).

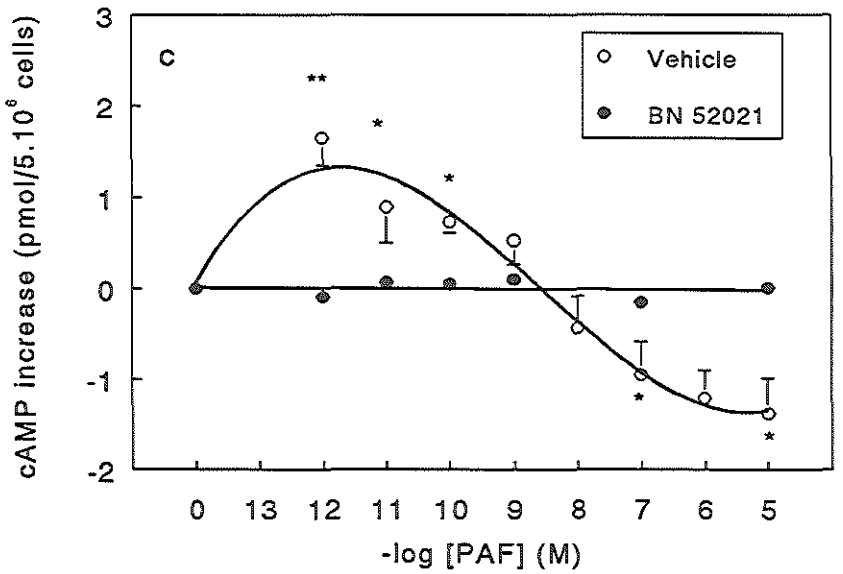
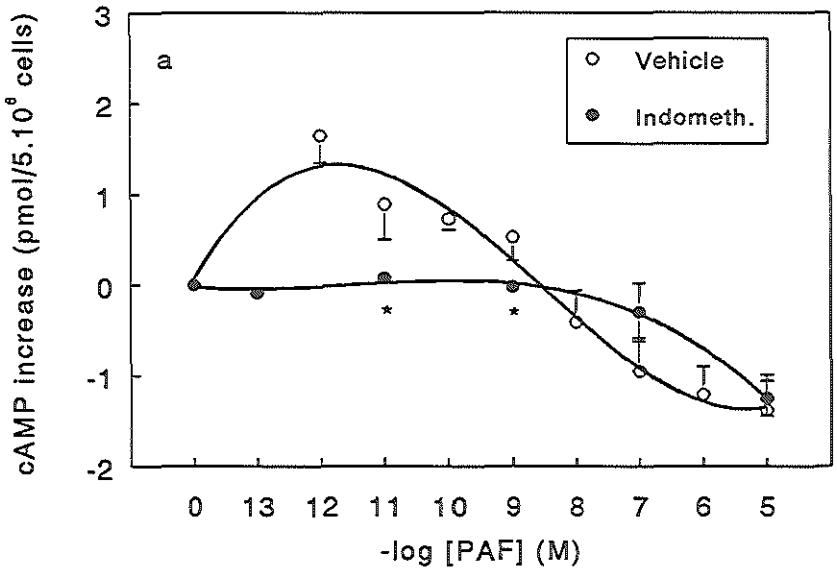


Figure 7.2. Effect of PAF in the absence (o) or presence (●) of BN 52021 (10 μM) on c-AMP response in naive (panel a, n=10), sensitized (panel b, n=6), and antigen challenged (panel c, n=10) AM. BN 52021 was added to the cellsuspensions 5 min prior to incubation with PAF. Data are expressed as mean absolute increases in c-AMP levels ± SEM. *P<0.01 ; **P<0.005, compared to values obtained with vehicle alone.

Since there are strong indications that PAF acts via the liberation and metabolism of arachidonic acid [5,8,21], we investigated the effects of indomethacin (a cyclooxygenase inhibitor) and AA 861 (a 5-lipoxygenase inhibitor) on the cyclic AMP- response of PAF in antigen challenged AM.
Figure 7.3.a shows that indomethacine (3 μM) completely blocked the cyclic AMP-

increasing effect of PAF (10^{-13}-10^{-10} M) in antigen challenged AM, whereas the cyclic AMP- decreasing effect of PAF (10^{-8}-10^{-5} M) remained unaffected.
On the other hand, AA 861 (10 µM) effectivily blocked the cyclic AMP- decreasing effect of PAF while the increasing cyclic AMP effect of PAF was unchanged (cf Figure 7.3.b).



7.2.a

7.5. discussion

In this report we describe the effects of Platelet Activating Factor (PAF) on cyclic AMP- production in alveolar macrophages (AM). PAF did not alter cyclic AMP- levels in naive AM but induced a biphasic cyclic AMP- response in both sensitized and antigen challenged AM. In these AM, low concentrations of PAF increased cyclic AMP- levels while higher concentrations of PAF decreased cyclic AMP- levels. Both effects of PAF could be blocked by the specific PAF- receptor antagonist BN 52021, indicating a receptor-mediated mechanism. Furthermore, the selective cyclooxygenase inhibitor indomethacin blocked the rise in cyclic AMP- levels of low PAF- concentrations without affecting the decrease in cyclic AMP- levels of high PAF- concentrations. This implicates that the rise in cyclic AMP is linked to the intracellular

generation of cyclooxygenase products. Pretreatment of AM with the selective 5-lipoxygenase inhibitor AA 861 did not affect the stimulatory effect of PAF on cyclic AMP- levels while the inhibitory effect of PAF was completely reversed. Therefore, it appears that the decrease of cyclic AMP- levels due to higher PAF- concentrations is mediated by products of 5-lipoxygenase.

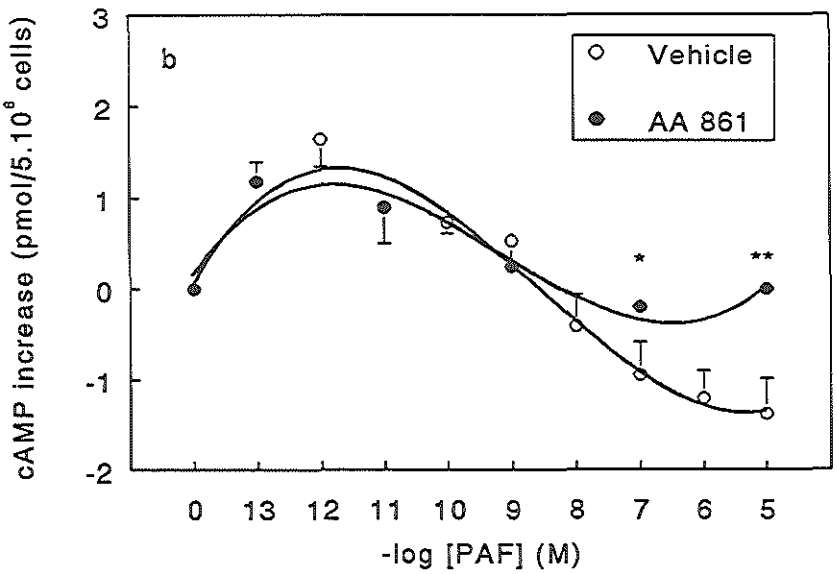


Figure 7.3. Effect of indomethacine (panel a) and AA 861 (panel b) on the c-AMP response of PAF in antigen challenged AM. Indomethacine (3 μM) or AA 861 (10 μM) was added to the cellsuspensions 5 min prior to incubation with PAF. Data are expressed as mean absolute increases of basal c-AMP levels ± SEM from 5 duplicate experiments. *P< 0.05; **P<0.005, compared to values obtained with vehicle alone.

These results show that PAF- receptor stimulation affects adenylyl cyclase activity not directly but via modulation of eicosanoid production. Indeed, Adolfs *et al.* [1] using peritoneal macrophages, have shown that indomethacin blocks the PAF- induced rise in cyclic AMP- levels. In addition, Bachelet *et al.* [2] have shown that agents, known to increase intracellular cyclic AMP- levels, antagonize the PAF- induced arachidonate release fron guinea pig alveolar macrophages.

The underlying mechanisms however, of the possible interactions between lipid mediators, like PAF and eicosanoids, and second messenger systems are still

unclear. In platelets and neutrophils, PAF has been shown to enhance PI- turnover resulting in the generation of IP_3 and DAG [16,17,21]. Furthermore, it was shown, using guinea pig AM that PAF can liberate arachidonic acid from the phospholipid membrane [2]. Probably, both PAF-effects are interrelated, as IP_3 mobilizes intracellular Ca^{2+}, necessary for phospholipase A_2 activity which in turn enhances the liberation of arachidonic acid. In addition, DAG- breakdown via lipases results in the generation of arachidonic acid [19].

As such, the liberated arachidonic acid is now susceptible to further conversion by cyclooxygenase and 5- lipoxygenase enzymes. Because both enzymes use the same substrate, they can be regarded competitors of other. In view of our results, low concentrations of PAF induce the conversion of arachidonic acid into mainly prostanoids, which in turn feedback on the cell to increase intracellular cyclic AMP-levels. At higher concentrations of PAF, sufficient intracellular Ca^{2+} has been mobilized by IP_3 to activate 5-lipoxygenase activity which, in contrast to cyclooxygenase, is a calcium- dependent enzyme [12]. Consequently, the high 5-lipoxygenase activity in the cell will lead to increased leukotriene- production at the expense of prostanoid- production. As leukotrienes do not affect cyclic AMP- levels in AM, the decreased production and release of prostanoids will result in decreased cyclic AMP-levels (as no clear indications are made in the available literature, we incubated AM with leukotrienes in a dose range of 10^{-8}-10^{-5} M, which did not alter intracellular cyclic AMP- levels (2.15 ± 0.09 - 2.20 ± 0.05 pmol/1·10^6 cells), unpublished observations).

Interestingly, PAF modulates cyclic AMP- levels only in sensitized and antigen challenged (i.e. immunological and anaphylactic) conditions. This is not only consistent with the commonly accepted idea that PAF is a potent mediator under bronchial hyperreactive conditions [9,18] but is also in agreement with our previous observation that sensitization and antigen challenge induce an improved coupling between stimulatory receptors and adenylyl cyclase [4]. Taken together, these observations imply that prostanoids, released by the action of PAF, can not modulate intracellular cyclic AMP- levels in naive AM, but become efficient stimulants of adenylyl cyclase in sensitized and antigen challenged AM.

As has been reported by others, minor alterations in intracellular cyclic AMP- levels can induce considarable changes in cellular activity. Superoxide production from neutrophils, as reported by Lad *et al.* [14] and monocytic expression of IL-1 activity [13] and the release of lysosomal enzymes from macrophages [23] are inhibited to a great

extend by only small increases of cyclic AMP- levels.
We therefore conclude that the dose- dependent PAF- induced changes in intracellular cyclic AMP- levels can induce significant changes in cellular activity of alveolar macrophages, which might contribute to the drastic pathophysiological changes as observed in bronchial anaphylactic reactions.

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chapter eight

differential eicosanoid release from alveolar macrophages induced by platelet activating factor; involvement of adenylyl cyclase *

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8.1. summary

The effects of platelet activating factor (PAF) on cAMP-production, PGE_2- and LTB_4-release was investigated in naive and antigen challenged guinea pig alveolar macrophages (AM). Basal cAMP-levels, PGE_2- and LTB_4-release from antigen challenged AM (ac-AM) were significantly enhanced as compared to naive AM. In addition, cyclic AMP-levels and LTB_4-release in naive AM were unaltered by PAF (10^{-12}-10^{-6}M) wherease PGE_2-release was slightly enhanced (plus 42 ng/5 $\cdot 10^6$ AM) by low concentrations of PAF (10^{-12} M). In ac-AM, low PAF-concentration (10^{-12} M) induced enhanced cAMP-production and PGE_2-release and decreased LTB_4-release. Higher PAF-concentrations (10^{-6} M) reversed these effects thus decreasing cAMP-levels and PGE_2-production and stimulating LTB_4-release. All PAF-induced effects were receptor-mediated as they could be reversed by pretreating AM with the PAF-antagonist BN 52021. Furthermore, pre-incubation of ac-AM with indomethacine reversed the PAF-induced rise in cAMP-levels and PGE_2-release while the LTB_4-release was unaffected. Similarly, pre-incubation with the lipoxygenase inhibitor AA 861 reversed the PAF-induced LTB_4-release while the cAMP-production and PGE_2-release remained the same. We conclude that PAF dose-dependently affects arachidonic acid metabolism which results in altered cAMP production in AM which may contribute to the worsening of pulmonary inflammation.

Key words: alveolar macrophages, platelet activating factor, arachidonic acid metabolism, prostaglandin E_2, Leukotriene B_4, cyclic AMP, adenylyl cyclase, pulmonary inflammation.

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8.2. introduction

Platelet activating factor (PAF) is a potent immuno stimulator released from a variety of cells known to be involved in pulmonary inflammatory reactions associated with asthma [1,8]. In turn, the activity of these pulmonary cells (e.g. eosinophils, basophils and alveolar macrophages) can be modulated by PAF, which renders this lipid mediator to act as an important amplifying mediator in the cascade of inflammatory events. In addition, PAF retains potent chemotactic activity [19,22,24,26] and induces the release of various immuno-modulators like leukotrienes [5,6,25], prostaglandins [12,15,25], and cytokines, such as IL-1 [23] and TNF [11] from different cells like AM.
With respect to the intracellular mechanisms underlying these PAF-induced phenomena, it appears that, depending on the celltype and concentration of PAF, different second messengers may be produced upon PAF-receptor stimulation. PAF-receptors have been shown to be coupled to phosphoinositide (PI) breakdown and release of calcium from internal stores [8,16,20]. In addition, both stimulatory [14] and inhibitory [17,27] effects on cAMP-production have been reported in different immune cells (e.g polymorphnuclear cells and platelets) following exposure to PAF. Cyclic AMP is known to modulate a number of different macrophage functions. Generally, enhancement of intracellular cAMP levels induce diminished phagocytosis and decreased release of oxygen radicals and lysosomal enzymes [6].
In guinea pig alveolar macrophages (AM), we recently showed a biphasic response of adenylyl cyclase activity following PAF-stimulation of antigen challenged AM (but not in naive AM). Thus, low PAF-concentrations (10^{-13}-10^{-10}M) increased cAMP levels whereas higher PAF-concentrations (10^{-8}-10^{-5}M) were inhibitory. In addition, the cAMP-stimulatory effect of PAF could be reversed by pretreatment with indomethacin (inhibiting cyclooxygenase) while the cAMP-inhibitory effect was reversed following pretreatment with AA 861 (inhibiting lipoxygenase) [3].
In order to ascertain whether the PAF-induced alterations in intracellular cAMP concentrations are mediated via differential release of eicosanoids, we studied the release of PGE_2 and LTB_4, the predominant arachidonic acid metabolites from naive and antigen challenged AM exposed to different concentrations of PAF.

8.3. methods

8.3.1. animals and sensitization

Male Hartley guinea pigs (weighing 300 -500 g) were used throughout the study. Naive animals were anaesthetized by i.p. injection of sodiumpentobarbitone, the trachea cannulated and bronchoalveolar lavage was performed by repeated lavages of 8 ml volumes of 0.9 % saline (total 150 ml). To obtain antigen challenged alveolar macrophages (ac-AM), animals were activily sensitized with ovalbumine as previously described [2]. Briefly, animals were sensitized by i.p. and s.c. injection of an ovalbumine solution (each 50 mg in 0.9 % sterile saline). Following a two weeks latent period, animals were subjected to a booster injection of ovalbumine after which the lavage was proceeded as described above.

8.4.2. isolation and preparation of alveolar macrophages

The lavage fluids were filtered through surgical gauze and centrifuged at 400 x g for 10 min at 4° C. Resuspension of the cellpellets in Gey Balanced Salt Solution (GBSS, pH 7.4) was followed by a Ficoll-Isopaque (Nycomed, Oslo, Norway)-gradient centrifugation (400 x g, 30 min, 4° C). After repeated washings, alveolar macrophages (AM) were resuspended in GBSS at a concentration of 3 $\cdot 10^6$/ml. Cytofuge preparations were stained with May Grünwald Giemsa staining. Viability of the AM-suspensions was tested by Trypan Blue exclusion and always exceeded 95 %.

8.3.3. incubation protocol

One ml samples (3 $\cdot 10^6$ cells) were incubated for 15 min at 37° C in the presence of 400 µM IBMX (3-isobutyl-1-methyl-xanthine, Janssen Chimica, Beerse, Belgium) and increasing doses of PAF (Sigma Chemicals, St. Louis, USA). When used, the PAF-antagonist BN 52021 (Ginkgolide B, a generous gift from Dr. P. Braquet, Le Plessis, Robinson, France) and inhibitors indomethacin or AA 861 (a selective 5-lipoxygenase inhibitor, kindly provided by Dr. S. Terao, Takeda Chemical Research Division, Osaka, Japan) were added to the AM-suspensions, 5 min prior to the incubation with PAF. After the incubation (15 min, 37° C), samples were centrifuged for 1 min and the cellpellet, separated from the cell free supernatant (SN), was resuspended in 150 µl Tris.HCl buffer (pH 7.4) and boiled for 3 min. Samples for LTB_4-determination were freeze-dried, resuspended in 250 µl methanol and, like SN for PGE_2-determination, stored at -80° C for further analysis.

8.3.4. eicosanoid- and cAMP-determination

PGE_2 and LTB_4 liberated in the SN were assayed using commercially available ELISA-kits (Cayman Chemical, Ann Arbor, USA). Detection limits for PGE_2 and LTB_4 were 3 and 1 pg/ml respectively. Cellular content of cAMP was determined by radioimmunoassay using [^{3}H] cAMP (Amersham, Amersham, UK) and a high-affinity binding protein as described previously [7].

8.3.5. data analysis

All data are expressed as the means ± standard error of the mean (S.E.M.). Statistical significance was evaluated by the unpaired Student's t test. A P-value < 0.05 was considered significant.

8.4. results

In table 8.1., basal cAMP-levels and spontaneous release of PGE_2 and LTB_4 from naive and ac-AM are presented. Basal cAMP-levels were significantly higher in ac-AM compared to naive AM (2.08 and 5.20 pmol/5 $\cdot 10^6$ AM respectively) but not affected by preincubation with either BN 52021, indomethacin (cyclooxygenase inhibitor) or AA 861 (5-lipoxygenase inhibitor). Similarly, spontaneous release of both eicosanoids was higher in ac-AM compared to naive AM. The basal PGE_2-release amounted in naive and ac-AM 109 and 503 pg/5 $\cdot 10^6$ AM respectively, whereas basal release of LTB_4, which was not detectable in naive AM, from ac-AM amounted 6.0 ng/5 $\cdot 10^6$ AM. As observed with cAMP-levels, preincubation with BN 52021, indomethacin or AA 861 did not affect spontaneous eicosanoid production in either AM-population. Figure 8.1. summarizes the data for PGE_2-release from naive AM after stimulation with increasing doses of PAF. As previously described [3], cAMP-levels in these naive AM were not affected by PAF (10^{-12}-10^{-6}M) nor by PAF following preincubation with indomethacin or AA 861. Incubation of these AM with a low dose of PAF (10^{-12}M) induced increased release of PGE_2 (plus 42 ng/5 $\cdot 10^6$ AM) which could be reversed by pretreatment with indomethacin (panel B) but not with AA 861 (panel C). At higher PAF-doses (10^{-9}-10^{-6}M), no change in the release of this metabolite was observed as compared to basal value (cf. Table 8.1.).

Table 8.1. Basal cAMP-levels and basal release of PGE_2 and LTB_4 from naive and antigen challenged AM.

	cAMP (pmol/5 $\cdot 10^6$ AM)	PGE_2 (ng/5 $\cdot 10^6$ AM)	LTB_4 (ng/5 $\cdot 10^6$ AM)
naive AM			
vehicle	2.08 ± 0.01	109 ± 3	n.d.
+ indomethacine (3 μM)	2.08 ± 0.02	121 ± 4	n.d.
+ AA 861 (10 μM)	2.11 ± 0.01	112 ± 3	n.d.
ac-AM			
vehicle	5.20 ± 0.19*	503 ± 24*	6.0 ± 0.2
+ BN 52021	5.15 ± 0.19*	477 ± 14*	5.5 ± 0.2
+ indomethacin (3 μM)	5.35 ± 0.12*	501 ± 11*	6.1 ± 0.1
+ AA 861 (10 μM)	5.26 ± 0.17*	546 ± 13*	5.5 ± 0.2

Abbreviations: ac-AM, antigen challenged AM; Data are expressed as means ± S.E.M. from 3 (naive) and 9 (ac-AM) duplicate experiments respectively; n.d.: not detectable; *: $p < 0.05$ as compared to corresponding value in naive AM.

The release of LTB_4 after PAF-incubation, either with or without preincubation with indomethacin or AA 861, was not detectable in naive AM.

Using ac-AM, striking differences were observed in the PAF-stimulated production of cAMP and the release of both PGE_2 and LTB_4 when compared to naive AM (figure 8.2.). To confirm our previous hypothesis that the PAF-induced alterations in intracellular cAMP-levels are mediated via the production and release of arachidonic acid metabolites, we determined, under the same conditions, the release of PGE_2 and LTB_4 from ac-AM using the same concentration range of PAF as desribed above.

As figure 8.2.a shows, incubation of ac-AM a low concentration of PAF (10^{-12}M) indeed resulted in an enhanced PGE_2-production (from 503 to 710 ng/5 $\cdot 10^6$ AM) whereas at a higher PAF-concentration (10^{-6}M) the release of PGE_2 was decreased from 503 to 352 ng/5 $\cdot 10^6$ AM. The enhanced PGE_2-release by PAF could be partially reversed with indomethacin while the reduced PGE_2-release remains unaffected. On the other hand, preincubation of ac-AM with AA 861 reverses the PAF-induced decrease in PGE_2-release whereas the increment in PGE_2-release remains unchanged. Finally, in the presence of BN 52021, PAF is unable to alter PGE_2-release.

Concidering the LTB_4-release, the results showed similar, though quite opposite results compared to PGE_2-release (figure 8.2.B). PAF at a concentration of 10^{-12}M decreased the release of LTB_4 from ac-AM (from 6.0 to 4.4 ng/5 $\cdot 10^6$ AM) whereas at the highest concentrations used (10^{-6}M), the release of this metabolite was

increased to 8.7 ng/5 $\cdot 10^6$ AM. The PAF-induced decrease in LTB_4-release was inhibited by pretreatment of ac-AM with indomethacin but not with AA 861. The increase in LTB_4-release by PAF was however not affected by indomethacin but could be completely blocked by pretreatment of ac-AM with AA 861. Again, the PAF-induced alterations in LTB_4-release were completely antagonized by BN 52021.

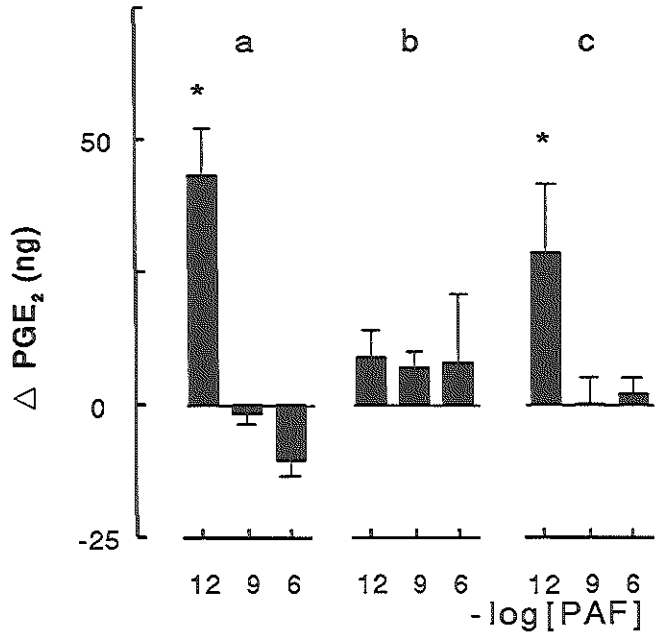

Figure 8.1. Absolute changes in PGE_2-release from naive AM following stimulation (15 min) with increasing doses of PAF (10^{-12}, 10^{-9} and 10^{-6}M). a: vehicle; b: in the presence of indomethacin (3 μM) and c: in the presence of AA 861 (10 μM). Amounts of LTB_4 released were below detection limit. Intracellular cAMP-levels were unaffected by any treatment. Data are expressed as means ± S.E.M. from 3 duplicate experiments. *: $P<0.05$ as compared to basal value.

In analogy with our previous findings [3], PAF (10^{-12}M) induced an increase in cAMP-level (plus 1.80 pmol/5 $\cdot 10^6$ AM) whereas PAF at a concentration of 10^{-6}M reduces cAMP-level by 0.94 pmol/5 $\cdot 10^6$ AM (cf. Fig 2C). Both PAF-induced alterations in cAMP-levels were receptor-mediated as they were completely blocked by pretreatment with the PAF-receptor antagonist BN 52021.

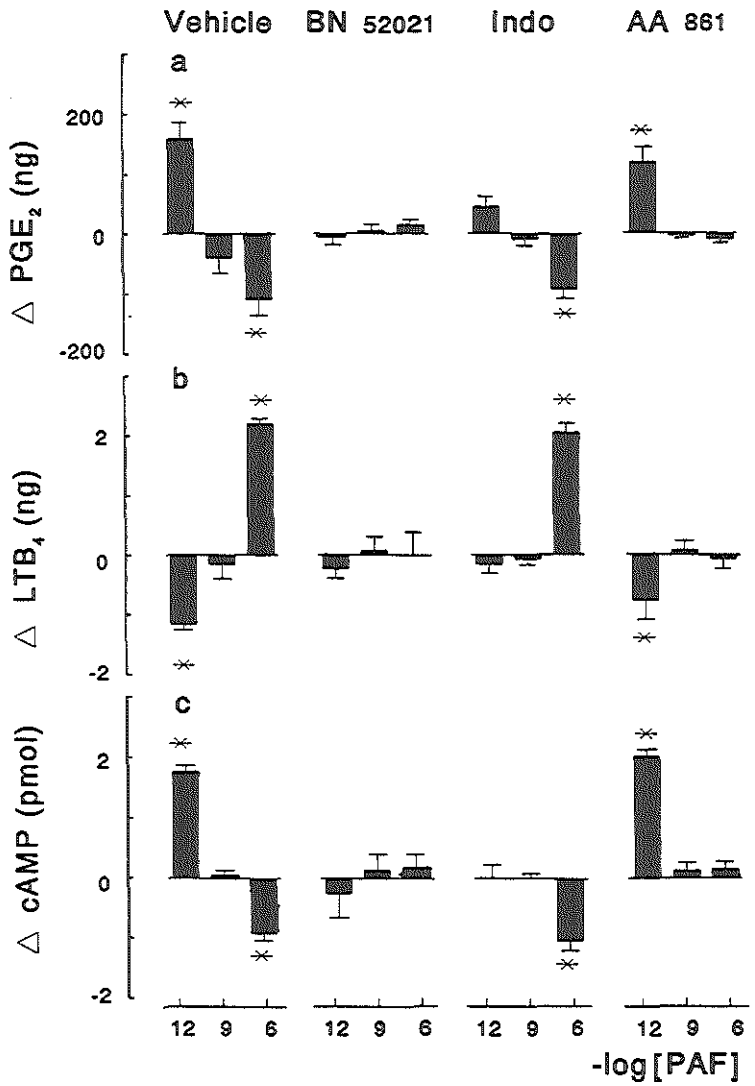

Figure 8.2. Absolute increases in cAMP-levels and PGE_2- and LTB_4-release from antigen challenged following stimulation (15 min) with increasing doses of PAF (10^{-12}, 10^{-9} and 10^{-6}M). *Abbreviations*: indo, indomethacine; a: PGE_2-release (ng/5 $\cdot 10^6$AM), b: LTB_4-release (ng/5 $\cdot 10^6$AM) and c: cAMP-concentrations (pmol/5 $\cdot 10^6$AM). Used drugs for pretreatment: BN 52021, 10 μM; indomethacin, 3 μM and AA 861 10 μM. Data are expressed as means ± S.E.M. from 9 duplicate experiments. *: $P<0.05$ as compared to basal value.

Furthermore, the cAMP-enhancing PAF-effect was cyclooxygenase dependent as it could be reversed by pretreatment of ac-AM with indomethacin (3 μM). On the other hand, the PAF-induced cAMP-decreasing effect could be regarded as lipoxygenase-dependent as preincubation of ac-AM with AA 861 (10 μM) reversed this effect of PAF.

8.5. discussion

The present data are in agreement with previous results and confirm our initial hypothesis that PAF-induced changes in cAMP production in AM indeed result from alterations in arachidonic acid metabolism [3]. In antigen challenged AM (ac-AM), both basal cAMP-levels and spontaneous PGE_2-release were substantially increased as compared to naive AM, suggesting that the spontaneous released PGE_2 induces, via feedback signals, cAMP production of the AM. Indeed, if ac-AM are exposed to 1 pM of PAF, which greatly enhances PGE_2-release, cAMP-levels are conversely enhanced. Similarly, reduction in PGE_2-release by 1μM PAF coincides with decreased intracellular cAMP production. After preincubation of the ac-AM with the selective cyclooxygenase inhibitor indomethacin, the response of low PAF-concentrations to induce PGE_2-release is impaired. Stimulation of adenylyl cyclase by PAF in this dose-range is abolished as well, which shows again that released PGE_2 is responsible for the PAF-induced increase in cAMP. As expected, stimulation of both PGE_2-release and cAMP production by these low concentrations of PAF were not affected by pretreating ac-AM with the selective lipoxygenase inhibitor AA 861, at least not in a direct fashion (see below). Exposure of ac-AM to much higher concentrations of PAF (10^{-6}M) results in a strong stimulation of LTB_4-release, which is not affected by pretreatment with indomethacin whereas the selective lipoxygenase inhibitor AA 861 completely eliminates this PAF-induced stimulation of LTB_4-release.
PAF has been shown to stimulate PI-turnover implicating that PAF-receptors are probably coupled to phosphatidylinositol hydrolysis to yield IP_3 and DAG and the subsequent release of Ca^{2+} from internal stores. In addition, both phospholipase A_2 and lipoxygenase are, in contrast to cyclooxygenase, Ca^{2+}-dependent enzymes. Therefore, in response to enhanced Ca^{2+}-concentrations, via PLC-activation by higher concentrations of PAF, arachidonic acid metabolism in AM is shifted towards lipoxygenase resulting in the generation of leukotrienes like LTB_4. Free arachidonic acid is only in limited amounts available for metabolism into various eicosanoids,

suggesting that enhanced leukotriene production simultaneously results in decreased prostanoid production. Indeed, as figure 8.2. shows, enhanced LTB_4-production by 10^{-6}M PAF coincides with a reduction in PGE_2-release compared to vehicle-value. As a result of impaired PGE_2-release (i.e. diminished feedback signal), the production of cAMP will be reduced as well. The reduced LTB_4-release after exposure of ac-AM to a low PAF-dose (10^{-12}M) can be explained by the preferential metabolism of the limited amount of free arachidonic acid into prostanoids (as apparently induced by this low PAF-dose).
In a similar way, the changes noted for LTB_4 and PGE_2-release by 10^{-12}M PAF after pretreatment with indomethacin can be explained. Pretreatment of AM with indomethacine results in impaired PGE_2-release and reversement of the decreased LTB_4-release by 10^{-12} M PAF. Furthermore, pretreatment of ac-AM with 10 μM AA 861 and subsequent incubation with 10^{-6}M PAF results in no net change in LTB_4-release and a return to basal values considering PGE_2-release and cAMP-levels.
It should be noted however that PAF modulates intracellular cAMP levels only in ac-AM, not in naive AM. As we have shown before, the adenylyl cyclase responsiveness to stimulatory agents (like PGE_2) is enhanced in these cells, probably due to an increased number of α_s-subunits of the G-proteins [4]. Though PAF is able to stimulate PGE_2-production in naive AM, the released amount of PGE_2 is apparently insufficient to stimulate cAMP production due to the relative poor coupling of receptors to adenylyl cyclase in these cells. In ac-AM however, PAF induces PGE_2-production which exceeds the production from naive AM which, together with the enhanced coupling between receptors and adenylyl cyclase, results in enhanced intracellular cAMP-levels.

Several reports have described similar biphasic responses by PAF. Dubois *et al.* [11] observed stimulation of TNF- and LTB_4-release from rat AM after exposure to 10^{-14} - 10^{-6}M PAF; the dose-response curve was bell-shaped with peak concentrations of 10^{-10}M. In addition, Pignol *et al.* [23] reported that PAF enhanced IL-1 release at low doses (10^{-12}-10^{-10}M), while at higher concentrations PAF was shown to be inhibitory. Though essential differences in cell origin and incubation procedures are present, we speculate that the release of both IL-1 and TNF by macrophages might be based on the PAF-induced modulation of arachidonic acid metabolism.
Though under normal physiological conditions only minor levels of PAF are circulating, pathogenic triggers like shock, anaphylaxis and antigens, might rapidly

elevate PAF-concentrations which lead to enhanced release of proinflammatory mediators. In this respect, it is noteworthy that PAF-release from AM and eosinophils is increased in asthmatics as compared to controls [13,19]. In view of our data, increased production and release of PAF from pulmonary cells, initially during anaphylactic reactions, may induce enhanced LTB_4-release from AM. Since PAF and LTB_4 are extremely chemoattractive agents for inflammatory cells [10,18,19,21,22,24,26], both mediators may act synergistically to intensify pulmonary inflammation.
In conclusion, PAF dose-dependently affects arachidonic acid metabolism in AM. Low concentrations induce the release of PGE_2 whereas higher concentrations induce the production of LTB_4. The latter effect may be of great relevance as a putative mechanism for amplification of pulmonary inflammation.

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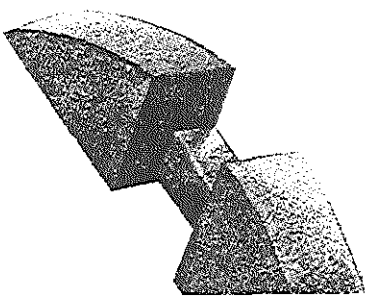
PART FOUR

ADENYLYL CYCLASE RESPONSIVENESS IN HUMAN ALVEOLAR MACROPHAGES



chapter nine

stimulation of cyclic amp-production in human alveolar macrophages induced by inflammatory mediators and ß-sympathicomimetics. a comparison between healthy subjects, patients with chronic obstructive pulmonary disease and asthmatics *

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9.1. summary

We have investigated the effects of inflammatory mediators and ß-adrenergic agonists on the adenylyl cyclase responsiveness in alveolar macrophages (AM) from control subjects, patients suffering from chronic obstructive pulmonary disease (COPD) and asthmatics. Basal cyclic AMP (cAMP) levels in AM from COPD patients were significantly elevated (plus 42%) as compared to controls. In addition, the adenylyl cyclase responsiveness to prostaglandin E_2, histamine and the ß-adrenergic agonists isoprenaline and salbutamol was significantly impaired in AM from COPD patients and asthmatics. The lipid mediator platelet activating factor (PAF) showed no effect on cAMP production in all three AM populations. Furthermore, the cAMP-enhancing effects of isoprenaline, salbutamol and histamine appeared to be mediated via $ß_2$-adrenergic and H_2-histaminergic receptor subtypes, respectively. Taken together, these data suggest an intrinsic desensitization phenomenon in AM from COPD patients and asthmatics.

Key words: alveolar macrophages, cyclic AMP, chronic obstructive pulmonary disease, asthma, adenylyl cyclase, inflammatory mediators, ß-adrenergic agonists

* F.D. Beusenberg, J.G.C. van Amsterdam, H.C. Hoogsteden, P.R.M. Hekking, J.W. Brouwres, H.P. Schermers, I.L. Bonta. Stimulation of cyclic amp-production in human alveolar macrophages induced by inflammatory mediators and ß-sympathicomimetics. A comparison between healthy subjects, patients with chronic obstructive pulmonary disease and asthmatics. (submitted for publication).

9.2. introduction

Pulmonary inflammation is incontrovertibly associated with chronic obstructive pulmonary disease (COPD) and asthma [10]. Within the complex pathophysiological processes, alveolar macrophages (AM) exert a predominant role. Firstly, these cells act as scavengers in the first line of host defense by means of phagocytosis and related features like the production and release of reactive oxygen radicals and lysosomal enzymes [14,32]. Secondy, AM retain a large potency to modulate the activity of other pulmonary cells via the release of inflammatory mediators like prostaglandins, leukotrienes and platelet activating factor [1,13,22,23,27] and cytokines like IL-1 and TNF-α [21,31].

With respect to functional activity between AM from controls, COPD patients and asthmatics, several conflicting results have been reported. Thus, eicosanoid release from AM of asthmatics and COPD patients has been shown to be impaired [17], unaltered [3] or even enhanced [9,12]. Furthermore, the release of PAF, oxygen radicals and ß-glucuronidase appears to be enhanced in AM from asthmatics [2,11,34].

Pulmonary mediators and other substances like hormones and drugs interact with cells via specific surface receptors coupled to transmembrane signalling systems which produce several second messengers [4]. Functional activity of AM (and cells in general) is largely associated with intracellular levels of cyclic AMP (cAMP), a second messenger produced by the action of adenylyl cyclase. In general, high levels of cAMP coincide with down-regulation of functional activity [7].

Though the effects of inflammatory mediators on general aspects of pulmonary inflammation have been clearly described, reports on their effects on AM remain scarce. Histamine, leukotrienes and PAF have potent bronchoconstrictive actions, stimulate mucus secretion and exert strong chemotactic potency for inflammatory cells [15,18,20,29,35]. Prostaglandins of the E-type have mainly bronchodilating properties [16]. To further investigate the role of mediators in pulmonary inflammation, we performed studies on the effects of histamine, prostaglandin E_2 (PGE_2), prostacyclin (PGI_2) and PAF on adenylyl cyclase responsiveness in human AM from controls, COPD patients and asthmatics. In addition, the effects of the ß-adrenergic agonists isoprenaline and salbutamol (frequently used as anti-asthmatic drug) on adenylyl cyclase responsiveness were determined as well.

9.3. methods

9.3.1. subjects

Smoking female volunteers (> 5 pack years, age 23-37 yrs, mean age 30) were studied. None of the subjects had a history of pulmonary disorders or received any medication two months prior to the study. Informed consent for bronchoalveolar lavage (BAL) was obtained. BAL was performed under general anesthesia using a fiberoptic bronchoscope. Four subsequent volumes of 50 ml sterile saline were instilled into a subsegmental bronchus of the right middle lobe, followed by gentle aspiration. The obtained BAL fluids were kept on ice untill further isolation of AM.

9.3.2. patients

The study included 12 COPD patients (7 female, 5 male, ages 24 to 75 years, mean age 51) and 2 non-atopic asthmatics (female, ages 31 and 37). Diagnosis of COPD patients (chronic bronchitis, n=6; bronchial emphysema, n=6) was based on history of clinical symptoms, chest X-ray and pulmonary function tests. Mean FEV_1 was 72 % ± 6.9 and mean FVC was 83 % ± 4.0 (percentage of predicted value). All of them were tobacco smokers (> 13 pack years), 3 received beta sympaticomimetics and 1 corticosteroids. Bronchial asthma was diagnosed on the basis of clinical history, severity of airway obstruction (FEV_1 less than 70 % of predicted value) and partial reversiblity after inhalation of beta adrenergic agonists.

9.3.3. isolation of alveolar macrophages

BAL-fluids were filtered through surgical gauze and centrifuged at 400 x g (10 min, 4° C). If necessary, erythrocytes were lysed by hypo-osmotic shock. The pellet was resuspended in Gey Balanced Salt Solution (GBSS), pH 7.4 and AM were purified by density gradient centrifugation (400 x g, 30 min, 4° C) using Ficoll-Isopaque (Nycomed, Oslo, Norway). After extensive washing, more than 95 % of the isolated cell-suspension consisted of AM as judged by May Grünwald Giemsa staining. Viability of the cells was assessed by dye exclusion using Trypan blue and AM suspensions with a viability exceeding 95 % were used for the experiments.

9.3.4. incubation procedure

One ml samples of AM (10^6) were incubated in GBSS at 37° C in the presence of 400 μM IBMX (3-isobutyl-1-methylxanthine, Janssen Chimica, Beerse, Belgium) with histamine (Pharmacy Department, Dijkzigt Hospital, Rotterdam, The Netherlands), prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), platelet activating factor (PAF), salbutamol or isoprenaline (all from Sigma, St-Louis, USA) dissolved in GBSS-buffer. In some experiments cell-suspensions were preincubated for 10 min with propranolol (Ciba-Geigy, Basel, Switzerland), cimetidine (Sigma, St Louis, USA) or mepyramine (Rhône-Poulenc, Paris, France). Following 15 min incubation, cells were spinned down and resuspended in 150 μl Tris-HCl buffer (pH 7.4) and boiled for 3 min. Cellular content of cAMP was determined by radioimmunoassay using [^{3}H] cAMP (Amersham, Amersham, UK) and a high-affinity binding protein as described previously [8].

9.3.5. statistical analysis

Data are expressed as means ± S.E.M. Statistical significance was evaluated by the unpaired Mann-Whitney U test. A p-value of < 0.05 was considered significant.

9.4. results

Table 9.1. shows the cellular differentiation of the BAL-fluids from three different groups of subjects (controls, COPD patients and asthmatics).

Table 9.1.: Cellular differentiation (in percentages) of BAL-fluids from controls, COPD patients and asthmatics.

	controls (22)	COPD patients (12)	asthmatics (2)
macrophages	95.5 ± 0.6	87.2 ± 3.6†	91.5 ± 0.5
lymphocytes	2.3 ± 0.4	4.3 ± 0.6†	3.5 ± 2.5
eosinophils	0.6 ± 0.2	1.9 ± 0.7†	3.2 ± 2.5
neutrophils	0.3 ± 0.1	4.8 ± 2.8†	0.4 ± 0.9
mononuclear cells*	1.3 ± 0.8	1.8 ± 0.6	1.4 ± 0.7

Number of observations in parenthesis; *, other than macrophages or lymphocytes; †, $p<0.05$ as compared to controls.

Alveolar macrophages make up the majority of the recovered cells (91.5-95.5%) with a marked decrease in the percentage in the BAL fluids from COPD patients and asthmatics and an increase in the number of lymphocytes, eosinophils and neutrophils.
Basal cAMP-levels were significantly higher (plus 42%) in AM from COPD patients as compared to control AM, while AM from (two) asthmatics showed the same level as controls (cf. Table 9.2.).

Table 9.2.: Basal cAMP levels in AM from controls, COPD patients and asthmatics.

	cAMP (pmol/10^6AM)
controls	1.63 ± 0.11
COPD patients	2.31 ± 0.28*
asthmatics	1.57 ± 0.93

Data are expressed as mean ± S.E.M. pmol/10^6 AM from 22 (controls), 12 (COPD patients) and 2 (asthmatics) duplicate experiments; *, $p<0.05$ as compared to controls.

The stimulatory effects of inflammatory mediators (PGE_2, PGI_2, PAF and histamine) and ß-sympathicomimetics (isoprenaline and salbutamol) on cyclic AMP production in different AM popultions are depicted in figures 9.1. and 9.2. Prostaglandin E_2 and histamine dose-dependently stimulate cAMP-production in the three AM-populations, though less effectively in AM from COPD patients and asthmatics (cf. Figures 9.1.a and 9.1.d respectively). Using PGI_2, no difference in adenylyl cyclase responsiveness among the three different AM populations (cf. Fig. 9.1.b) was observed. The lipid mediator PAF does not affect cAMP levels in AM (cf. Fig 9.1.c). At the lowest concentration (10^{-12}M) however, PAF tends to enhance cAMP production in AM from COPD patients (plus 15%) and asthmatics (plus 17%).
The adenylyl cyclase responsiveness to isoprenaline and salbutamol show similar results as the response of the inflammatory mediators. The non-selective ß-adrenergic agonist isoprenaline enhances cAMP levels in all three AM populations with the same potency (cf. Fig 9.2.a), whereas the response to the $ß_2$-selective adrenergic agonist salbutamol is largely reduced in AM from COPD patients and asthmatics (cf. Fig 9.2.b).

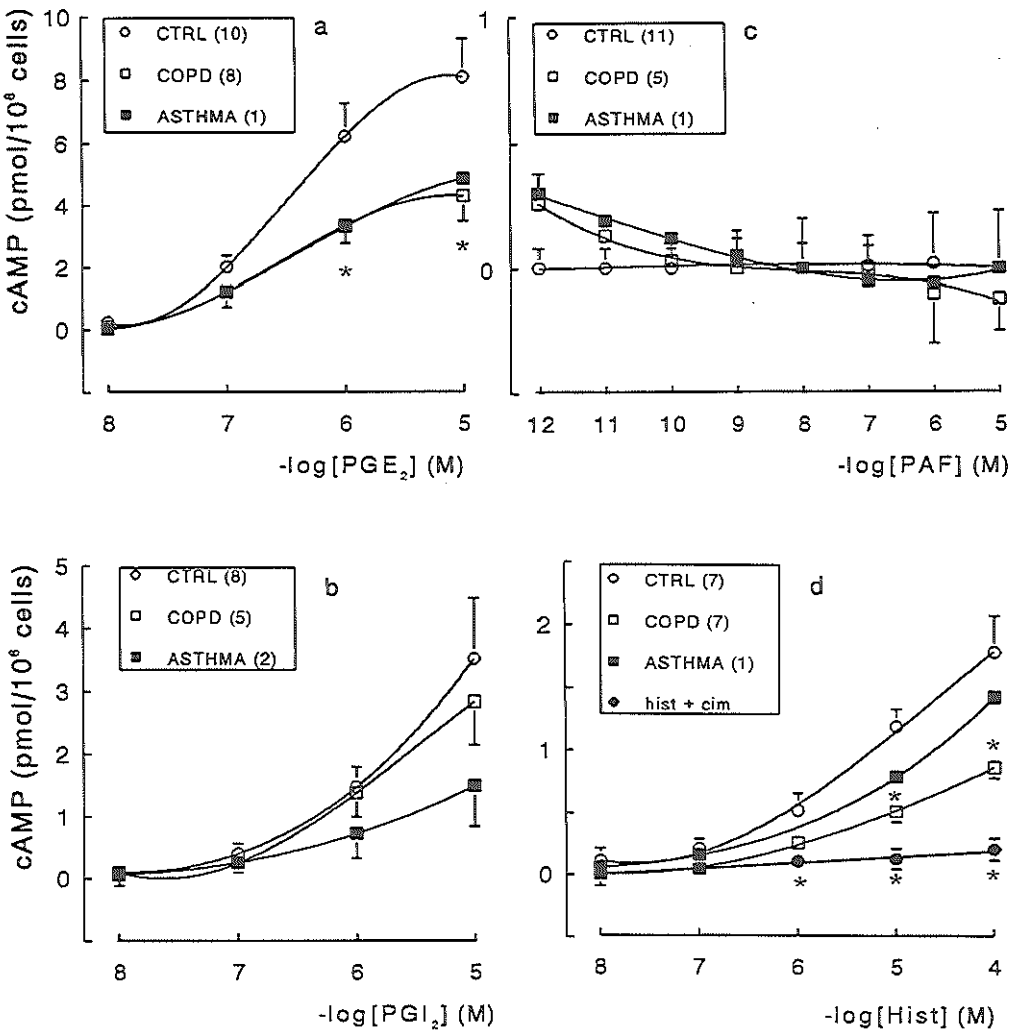

Figure 9.1. Cyclic AMP-production in AM from control subjects (CTRL, open circles), COPD patients (COPD, open squares) and asthmatics (ASTHMA, closed squares) following a 15 min. incubation with inflammatory mediators PGE_2 (a), PGI_2 (b), PAF (c), and histamine (d) in the presence of 400 μM IBMX. Prior to incubation with histamine, control AM were incubated with the H_2-selective antagonist cimetidine 10^{-5}M (panel d, closed circles). Number of duplicate observations in parenthesis. Data are expressed as mean absolute increase above basal cAMP-level ± S.E.M. (pmol/10^6 AM). *: $p < 0.05$ as compared to controls.

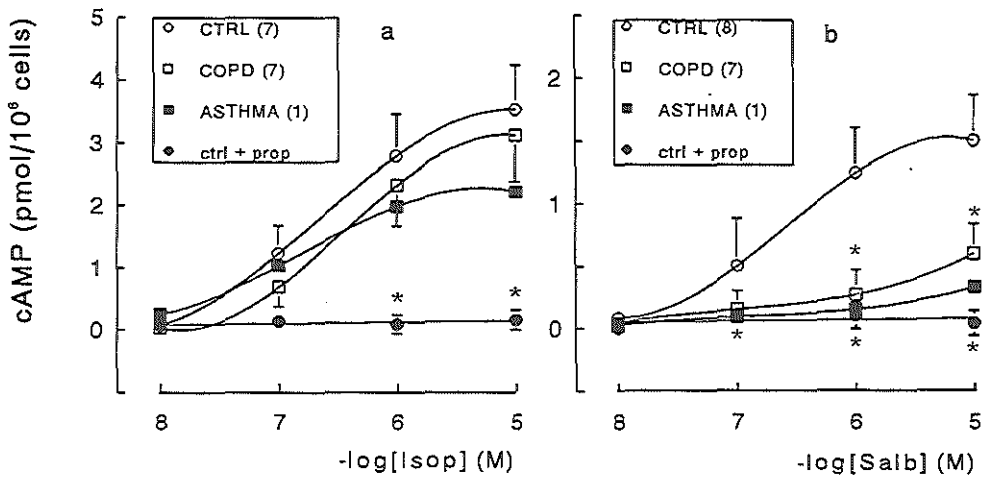

Figure 9.2. Cyclic AMP-production in AM from control subjects (CTRL, open circles), COPD patients (COPD, open squares) and asthmatics (ASTHMA, closed squares) following a 15 min. incubation with the ß-adrenergic agonists isoprenaline (Isop, panel a) and salbutamol (Salb, panel b) in the presence of 400 μM IBMX. Prior to incubation with the agonists, control AM were incubated with the non-selective ß-adrenergic antagonist propranolol (prop, 10^{-5}M, closed circles). Number of duplicate observations in parenthesis. Data are expressed as mean absolute increase above basal cAMP-level ± S.E.M. (pmol/10^6 AM). *: $p < 0.05$ as compared to controls.

The stimulatory effects of the isoprenaline and salbutamol on cAMP production were completely blocked by propranolol 10^{-5}M (cf. Fig. 9.2.a and 9.2.b). Considering the high potency of salbutamol, $ß_2$-subtype adrenergic receptors appear to mediate this action. The histaminergic effect is attained by stimulation of H_2-histaminergic receptors as the H_2-selective antagonist cimetidine (10^{-5}M) completely reversed the effect (cf. Fig. 9.1.d) while the H_1-selective antagonist mepyramine (10^{-5}M) was in this respect not effective (results not shown).

It should be mentioned that due to the poor availability of AM from asthmatics, we were not able to determine the adenylyl cyclase responsiveness more adequatly in this AM population.

9.5. discussion

Recently, we have shown in human alveolar macrophages that the $ß_2$-adrenergic agonist salbutamol and the phosphodiesterase inhibitor IBMX inhibited PGE_2-release and stimulated LTB_4-secretion via enhancement of intracellular cAMP levels [6]. Hence, we were interested to determine whether inflammatory mediators and ß-sympathicomimetics showed the same potency to stimulate cAMP production in AM from controls, COPD patients and asthmatics.

Cellular composition of BAL fluids from the three groups of subjects differed largely with respect to the number of AM, eosinophils, neutrophils and lymphocytes which is in accordance with previous results. Thus, an increase in the number of predominantly eosinophils in BAL fluids from asthmatics has been reported by Wardlaw *et al.* [36]. and Tomioka *et al.* [33] wherease in BAL fluids from COPD patients an increase of neutrophils has been reported [24]. The increase in the number of lymphocytes in COPD patients and asthmatics (both groups consisting of non-allergic subjects) observed by us and others [19,25] suggests that these cells, besides AM, eosinophils and neutrophils, may play an important role in pulmonary inflammation.

Present results show that initial basal cAMP levels differed largely between the AM populations. AM from COPD patients contained some 40% more cAMP than control AM whereas basal cAMP levels in AM from asthmatics showed no differences to controls.

For different reasons, it is difficult to interprete our data on basal cAMP in AM from asthmatics with functional parameters as conflicting data have been reported. Thus, while some reports indicate altered functional activity (e.g. chemiluminescence and arachidonic acid metabolism) in AM from asthmatics [11,12,17] others have reported similar results between AM from asthmatics and controls [3]. In addition, our results were merely based on 2 asthmatics (which suggested no differences in basal cAMP levels). Whether functional activity of AM from asthmatics indeed differs from controls remains to be established.

One can only speculate about the origin of the enhanced basal cAMP levels in AM from COPD patients. Possibly, the persistent local inflammatory environment generates various mediators, like PGE_2, PGI_2 and histamine which stimulate cAMP production in AM located in the alveolar compartment. Like in other tissues and cells [26,30], such continuous exposure of AM to inflammatory mediators, which may stimulate adenylyl cyclase, will ultimately induce a desensitization of the stimulatory

receptors. Consequently, AM will become less susceptible to respond to these inflammatory substances. The present data on the diminished responsiveness of the adenylyl cyclase coupled signal transduction system to various mediators and drugs suggest a heterologous desensitization phenomenon. The differences in potency between the ß-adrenergic agonists isoprenaline and salbutamol to stimulate adenylyl cyclase is in accordance with previous results. Using guinea pig AM, we suggested that the observed differences ara probably due to the partial agonistic effect of salbutamol [5].

The few data on AM from asthmatics presented here point to a similar desensitization of the adenylyl cyclase system in AM, suggesting that asthma shares some common immunoregulatory- and inflammatory related mechanisms with COPD.

Within the pathophysiology of asthma and COPD, data on the phenomenon of desensitization in pulmonary cells are limited and have been confined to mainly bronchial smooth muscles and blood leukocytes. It is suggested that diminished beta-adrenoceptor function in asthmatics is probably a consequence of the active disease state (following allergen challenge) rather than an intrinsic component of asthma [28]. In the present report however, we present evidence that a general (including beta-adrenergic) dysfunction of the adenylyl cyclase system in AM, an important cellular component within the pulmonary compartment, is a general and intrinsic feature of pulmonary inflammation associated with asthma and COPD.

Physiologically, the impaired responsiveness of the adenylyl cyclase system to stimulatory agents in AM from COPD patients and asthmatics would implicate that cellular functions of the AM which are affected by alterations in cAMP levels (like oxygen radical and enzyme production) are less susceptible to modulation by external factors. Whether this renders the AM more sensitive to external modulation via mechanisms distinct from the adenylyl cyclase pathway remains to be investigated.

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chapter ten

cyclic amp-enhancing anti-asthmatic drugs promote the production of leukotriene b_4 by human alveolar macrophages *

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10.1. summary

The modulatory effects of inducers of cAMP- and cGMP- production on PGE_2- and LTB_4- release were evaluated in two populations of human alveolar macrophages. The phosphodiesterase-inhibitor methyl-isobutylxanthine induced a 70% increase in cAMP, while the $ß_2$-selective agonist salbutamol induced a further increase of 100%. The latter stimulation was mediated via ß-adrenoceptors, as it was completely inhibited by pretreatment with propranolol. Elevation of cAMP- levels in alveolar macrophages by the combination (salbutamol + IBMX) resulted in inhibition of PGE_2-release (34%) and stimulation of LTB_4- release (58%). In control alveolar macrophages, the PGE_2-secretion was inversely correlated with intracellular cAMP-levels, while LTB_4 was not. In alveolar macrophages obtained from COPD- patients, basal cAMP- levels, and PGE_2- and LTB_4- release were all decreased (40%, 48% and 23% respectively) as compared to controls. A poor dependency of PGE_2- release on cAMP and no relationship between LTB_4 and cellular cAMP-levels is obtained in these cells. A large increase (130%-145%) in cGMP- production was attained in both alveolar macrophages populations using sodium nitroprusside (SNP), but appeared not to correlate with either PGE_2- or LTB_4 production. These results suggest that increased cAMP- levels by methyl-xanthines and/or salbutamol decrease the production of PGE_2 and indirectly stimulate alveolar macrophages to release the chemotactic mediator LTB_4, which may be potentially important in the induction of bronchial inflammatory reactions.

Key words: alveolar macrophages, cyclic AMP, eicosanoids, salbutamol.

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10.2. introduction

Inflammation of the airways is a characteristic feature of both asthma and chronic obstructive pulmonary diseases (COPD) which result from still largely unknown pathophysiological events [8]. Alveolar macrophages (AM), abundantly present throughout the respiratory tract, exert a number of different inflammation-related functions.

In the first line of host defence, AM efficiently eliminate micro-organisms via phagocytosis, the release of reactive oxygen intermediates and lysosomal enzymes [14,40]. Secondly, AM retain a large potency to modulate the activity of other cells via the release of cytokines, prostaglandins, leukotrienes and platelet activating factor (PAF) [2,37]. The released eicosanoid mediators, notably LTB_4 [5,13,26,27] and PGE_2 [17,30], may affect via feedback mechanism(s) the macrophage activity itself [23,29,35], or by interaction with other cells induce a myriad of immune-reactions [3]. LTB_4 is for instance chemotactic for mast cells [21], eosinophils [10] and neutrophils [28]. Thus, alveolar macrophages may be regarded as regulatory cells in inflammatory responses in COPD and asthma [39]. Interestingly, upon stimulation AM from asthmatic patients release greater amounts of inflammatory mediators, than AM derived from healthy subjects [9,20]. Undoubtly, generation and release of lipid mediators (prostaglandins, leukotrienes and PAF) by AM have important implications for the micro-environment within the bronchoalveolar area.

To which extend and way AM exert modulatory effects depend on various factors like basal intracellular cyclic AMP (cAMP) -level, state of priming and exposition to biologically active substances. Salbutamol and methylxanthine-derivatives like theophylline are frequently used in the treatment of asthma. Their benificial effect seems to be confined to relaxation bronchial smooth muscle via increasing intracellular cAMP-level. Information is lacking, whether their therapeutic effect is related to an additional action on other pulmonary cells, known to be involved in airway inflammation.

Like bronchial smooth muscle tone, intracellular cAMP-level is one of the factors ruling macrophage activity: high cAMP-levels generally coincide with low cellular activity [6]. The role of cyclic GMP (cGMP) is in this respect less clear. A limited number of reports indicate that this cyclic nucleotide may be implicated as a second messenger in a number of stimulatory immunological actions. Phagocytic stimulation of peritoneal macrophages by zymosan elicits a rapid rise in both cAMP- and cGMP-

formation [34,38] and soluble stimulators of guanylyl cyclase like muramyl dipeptide [36], sodium nitroprusside [34], PGE_2 [33] and somatostatin in the low dose-range [15] or exogenous cGMP [11] have been shown to enhance macrophage activity [15,33,36]. On the other hand, others do not find any macrophage stimulation by inducers of cGMP production [34].
In the present report, we investigated whether agents which enhance cyclic nucleotide levels, affect eicosanoid production in human AM from COPD- patients and smoking control subjects.

10.3. methods

10.3.1. subjects

Ten volunteers (female, age 25-40 yrs, mean age 31, all smokers) were studied. None of these subjects had a history of pulmonary disorders, nor did they receive any medication two months prior to the study. Informed consent for bronchoalveolar lavage (BAL) was obtained. Alveolar cells were obtained by BAL using a fiberoptic bronchoscope. Four subsequent volumes of 50 ml sterile saline were instilled into a subsegmental bronchus of the right middle lobe, followed by gentle aspiration. BAL fluids were subsequently kept on ice.

10.3.2. patients

Lung tissue was obtained from COPD- patients who had undergone thoracotomy for a small peripheral bronchial carcinoma. According to the criteria of the American Thoracic Society [1] all patients were diagnosed for COPD. Mean FEV_1 was 65.7 %, mean FVC was 87.3 % (calculated from normal predicted value). Within 30 min after surgical resection tissue was immersed in ice cold Krebs-Henseleit buffer (pH 7.6). AM were recovered by *in-vitro* lavage of peripheral airways with the same buffer using a 20 ml syringe.

10.3.3. isolation of alveolar macrophage

It was previously reported that the quality of the cell-fraction, concerning the percentage and viability of different cells in the population, is not dependent on the

method of isolation (*in-vivo* vs. *in-vitro* BAL)[19]. The obtained BAL-fluids from subjects and patients were filtered through surgical gauze and centrifuged at 400 x g (10 min, 4°C). If necessary, erythrocytes were lysed by hypo-osmotic shock. The pellet was resuspended in Gey Balanced Salt Solution (GBSS), pH 7.4 and AM were purified by gradient centrifugation (400 x g, 30 min, 4°C) on Ficoll-Isopaque (Nycomed, Oslo, Norway) and the resultant AM layer was washed twice. After this step more than 95 % of the cell-suspension consisted of AM as judged by May Grunwald Giemsa staining of cyto-centrifuge preparations. The viability of the cells was assessed by dye exclusion test using Trypan blue; only AM suspensions with a viability exceding 95 % were used for the experiments.

10.3.4. incubation procedure

One ml samples of AM ($2x10^6$/ml) were incubated in GBSS buffer at 37°C in the absence or presence of 1 mM IBMX (3-isobutyl-1-methylxanthine, Janssen Chimica, Beerse, Belgium) with salbutamol (Sigma, St-Louis, USA) and sodium nitroprusside (Merck, Darmstadt, FRG), dissolved in GBSS-buffer. In some experiments cell-suspensions were preincubated for 15 min with propranolol (Ciba-Geigy, Basel, Switzerland). Sixty minutes later AM were spinned down and the cell-free supernatant (SN) was removed and stored at -80°C for further analysis. Pellet was resuspended in 150 µl Tris- HCl- buffer (pH 7.4) and boiled for 3 min.

10.3.5. PGE_2-, LTB_4-, cAMP- and cGMP- determination

PGE_2 and LTB_4 liberated in the SN were assayed using commercially available ELISA-kits (Cayman Chemical, Ann Arbor, USA). Detection limits for PGE_2 and LTB_4 were 3 and 1 pg/ml respectively. Cellular content of cAMP was determined by radioimmunoassay using [^{3}H] cAMP (Amersham, Amersham, UK) and a high-affinity binding protein as described previously [7]. Cellular cGMP-levels were determined by RIA. Specific antibodies were obtained from rabbits immunized with 2'-O-succinyl-cGMP (Sigma, St-Louis, USA) covalently linked to BSA, using 1-ethyl-3-[3-dimethyl-aminopropyl] carbodiimide (Sigma, St-Louis, USA) as coupling agent, and used in a 4000-fold dilution in the RIA. [^{125}I]-iodinated succinyl-cGMP tyrosine methyl ester, purified by Sep-Pak C18 (Waters Associates, Milford, USA) column chromatography [43] was used as radioactive tracer. Prior to incubation in the RIA,

samples were acetylated according to the method described by Harper and Brooker [18]. Using this assay cGMP concentrations as low as 1.0 fmol can be determined.

10.3.6. statistical analysis

Data are expressed as means ± S.E.M. Statistical significance was evaluated by the Mann-Whitney U test. A p value of < 0.05 was considered significant.

10.4. results

Analysis of the cellular composition of BAL-fluids, performed before density gradient-centrifugation, showed large differences between controls and COPD subjects (cf. Table 10.1.). BAL-fluids of controls contained mainly alveolar macrophages (95%), whereas in BAL-fluids obtained from COPD-patients considerable numbers of eosinophils (7.4 %), neutrophils (14.4 %) and lymphocytes (10.6 %) were present.

Table 10.1. Cellular composition (in percentages) of BAL-fluids from control subjects and COPD-patients.

celltype	controls (n=10)	COPD patients (n=9)
macrophages	95.3 ± 1.0	66.0 ± 5.5†
eosinophils	0.5 ± 0.3	7.4 ± 2.2†
neutrophils	0.3 ± 0.2	14.4 ± 3.1†
lymphocytes	3.7 ± 1.0	10.6 ± 2.3†
mononuclear cells*	0.2 ± 0.1	1.6 ± 0.3†

Number of observations in parenthesis; data are expressed as means ± S.E.M.; *: other then macrophages or lymphocytes; †: $p<0.05$ as compared to control subjects.

Data on basal values and effects of different effectors on cyclic nucleotide levels and eicosanoid-release in control AM are summarized in Table 10.2. Basal cAMP- and cGMP- levels per 10^6 AM in the controlgroup were 1.36 pmol and 8.41 fmol respectively, reflecting a 160-fold difference in concentration between both nucleotides. In the presence of the nonselective phosphodiesterase (PDE) inhibitor IBMX, the cyclic nucleotide levels rose to 194% and 177% respectively of basal value for cAMP and cGMP.

Basal PGE_2-release amounted 120 pg/10^6 AM, but decreased in the presence of IBMX by some 25 % to 90 pg/10^6 AM. LTB_4-release was affected by IBMX to the

same degree as PGE_2, though in an opposite way (from 19.2 to 24.2 pg/10^6 AM). The combination of IBMX and the selective $ß_2$- adrenergic agonist salbutamol more efficiently enhanced intracellular cAMP-levels (180 % increase) than IBMX alone, but compared to IBMX PGE_2-release was only slightly inhibited by this combination. Considering stimulation of LTB_4-release, the combination of salbutamol and IBMX was twice as effective as IBMX alone. Propranolol (10^{-5} M) completely blocked all noted effects of salbutamol, as in the presence of this ß-adrenoceptor blocker it did not alter either PGE_2- and LTB_4-release, or cAMP-levels (no significant differences from basal values determined in the presence of IBMX alone). Intracellular cGMP-levels were not affected by salbutamol.

Table 10.2. Cyclic nucleotide levels and eicosanoid release in control human AM.

agent	cyclic AMP	cyclic GMP	prostaglandin E_2	leukotriene B_4
	pmol (%)	fmol (%)	ng (%)	ng (%)
A	1.36±0.14 (100)	8.41±1.13 (100)	120±5 (100)	19.2±4.2 (100)
B	2.70±0.29 (194±9)*	12.96±1.69 (177±19)*	90±6 (79±4)*	24.2±3.5 (138±9)*
C	4.04±0.40 (260±38)†	13.31±2.27 (170±9)	80±3 (65±4)†	30.4±3.1 (161±8)†
D	2.48±0.23 (199±7)‡	11.45±1.61 (167±12)	95±5 (77±6)‡	19.5±5.3 (101±7)‡
E	2.16±0.25 (178±9)ns	24.27±2.85 (312±61)†	98±6 (81±5)	25.4±5.1(133±10)

Abbreviations: A, saline; B, IBMX (1mM); C, IBMX (1mM) + salbutamol (10 µM); D, IBMX (1mM) + salbutamol (10µM) + propranolol (10 µM) and E, IBMX (1mM) + sodium nitroprusside (1mM). Data are expressed as means ± S.E.M. (per 10^6 AM) from 8-10 duplicate experiments. *, $p<0.05$ as compared to saline value; †, $p<0.05$ as compared to IBMX alone; ‡, $p<0.05$ as compared to value obtained in the presence of IBMX and salbutamol; ns, not significant from IBMX alone.

As the data in table 10.2. suggested a relationship between intracellular cAMP-levels and PGE_2- and LTB_4-release in control AM, we plotted the individual data of cAMP against release of PGE_2 and LTB_4 (cf Fig. 10.1.). Indeed PGE_2-release is dose-dependently under control of intracellular cAMP-level (regression-coefficient of 0.74), though in an inverse way: one pmol increase in intracellular cAMP reduces PGE_2-release by some 1.1 pg. In contrast to PGE_2, the release of LTB_4 is hardly dependent on the cellular level of this nucleotide as a poor correlation was calculated from these data (regression- coefficient of 0.15). On the other hand there was a clear relationship

between the decrease in PGE_2 secretion and the increase in LTB_4 release when the release of these mediators was compared within the same subject (cf. Fig. 10.2.). Plotting the data in a similar way for cGMP did not result in any correlation (data not shown).

The soluble guanylyl cyclase stimulator sodium nitroprusside (SNP) induces a steady increase in the cellular cytosolic cGMP concentration (180 % increase compared to IBMX alone), but only sligthly affects the cAMP- content of the cell (a non-significant 12 % decrease). Apparently, no cGMP-sensitive cAMP-hydrolyzing PDE [4] is involved in cellular regulation of cyclic nucleotide- metabolism in AM. Despite its effect on cGMP, no significant alterations in eicosanoid-release could be observed, indicating that an increase in this cellular cGMP-pool of control AM is, at least for PGE_2- and LTB_4 release, not relevant.

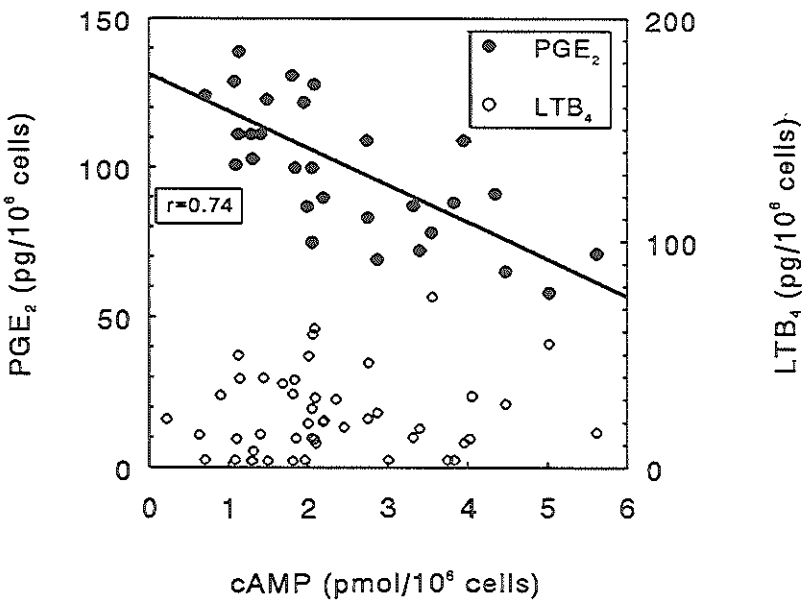

Figure 10.1. Correlation between cAMP-levels and PGE_2/LTB_4-release in control human AM. Cyclic AMP-, PGE_2- and LTB_4-values were determined as described in 'methods'. The PGE_2-values (closed circles) are depicted on the left Y-axis whereas the LTB_4-values (open circles) are depicted on the right Y-axis.

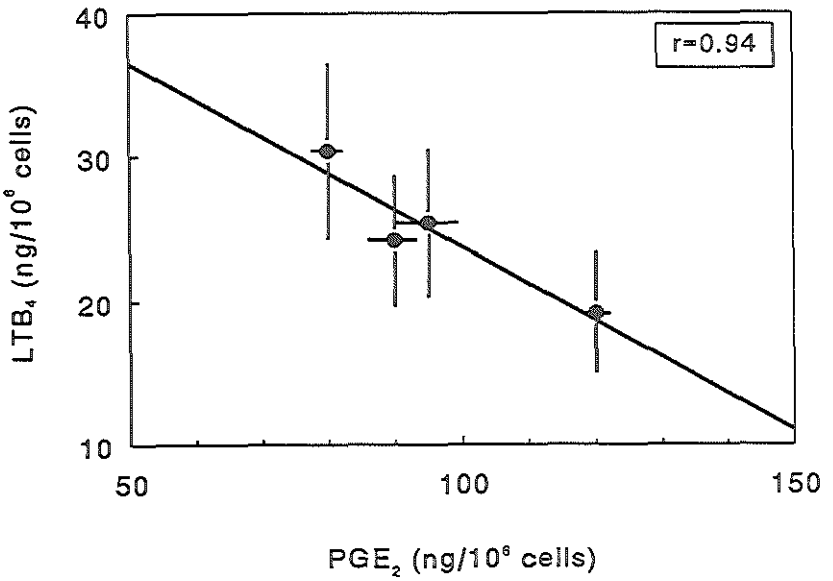

Figure 10.2. Correlation between PGE_2 and LTB_4-release in control human AM. Data are expressed as means ± S.E.M. The data were obtained from 10 duplicate experiments in which the PGE_2-values were correlated with the LTB_4-values within the same subject.

The same effectors were used in a parallel study using AM from COPD- patients (refered to as COPD-AM). In general, similar results were obtained with some notable exceptions (cf Table 10.3.). Unstimulated COPD-AM contained the same amounts of cGMP as control AM, but basal cAMP-levels were significantly decreased by some 30% and basal PGE_2- and LTB_4-release was greatly reduced in COPD-AM (47% and 23% reduction respectively compared to control AM).

Because of the low basal eicosanoid-release in COPD-AM, the effects of adenylyl cyclase inducers could be less well determined and cAMP-levels did not correlate with either PGE_2- or LTB_4- release (not shown). In the presence of IBMX, which enhanced the intracellular level of both cyclic nucleotides, PGE_2-release was inhibited to 17 % of the basal value. Lowering the concentration of IBMX was not possible, as IBMX- concentrations lower than 1 mM unadequatly protects cyclic nucleotide hydrolysis. LTB_4-release was slightly enhanced by IBMX (24 %). The combination of salbutamol and IBMX again increased cAMP-levels whereas no differences were

observed in PGE_2- and LTB_4- release, when compared to the effect of IBMX alone. Like in control AM, SNP significantly increased cGMP- levels in COPD-AM while cAMP- levels were unaffected. In addition, no changes in PGE_2- and LTB_4- release, as compared to the effect of IBMX alone, could be determined using SNP.

Table 10.3. Cyclic nucleotide levels and eicosanoid release in human AM from COPD patients.

agent	cyclic AMP	cyclic GMP	prostaglandin E_2	leukotriene B_4
	pmol (%)	fmol (%)	ng (%)	ng (%)
A	0.95±0.10 (100)	9.06±1.33 (100)	63±9 (100)	14.8±0.5 (100)
B	1.47±0.17 (155±8)*	15.95±1.93 (176±33)*	11±2 (17±3)*	18.3±2.0 (124±10)*
C	2.53±0.30 (265±21)†	13.57±2.27 (150±39)	9±3 (14±4)	16.2±0.3 (114±9)
E	1.45±0.17 (151±12)	37.36±2.43 (410±54)†	6±3 (10±3)	17.2±0.8 (117±9)

Abbreviations: A, saline; B, IBMX (1mM); C, IBMX (1mM) + salbutamol (10 μM) and E, IBMX (1mM) + sodium nitroprusside (1mM). Data are expressed as means ± S.E.M. (per 10^6 AM) from 7-9 duplicate experiments. *, $p<0.05$ as compared to saline value; †, $p<0.05$ as compared to IBMX alone;

10.5. discussion

When the basal values for cAMP-levels and eicosanoid-release are considered, some interesting differences between the macrophage populations of controls and COPD-patients are present. Basal levels of cAMP and PGE_2- and LTB_4-release were all reduced in COPD-AM compared with control AM. Interpreting the relevance of these differences is difficult since COPD-AM are obtained from a rather heterogenous group of COPD- patients with small peripheral bronchial carcinomas and a variety of medication. In addition COPD-AM are recovered from an environment characterized by frequent unspecific inflammatory reactions, which may influence the behaviour of these macrophages. Indeed, analysis of cyto- centrifuge preparations of the BAL-fluids from these patients revealed a large percentage of granolocytes and lymphocytes (cf Table 10.1.).

It has been described before, that smoking impaires the generation

ofleukotrienes [16,24,25,42], prostaglandins and thromboxane B_2 [24]. Lesions in arachidonic acid metabolism [24] and defects in 5-lipoxygenase activity [42] in alveolar macrophages elicited by smoking behaviour have therefore been suggested. This effect of smoking can however be excluded from our data, as smoking COPD-patients were compared with smoking control subjects.

In addition, we determined the effects of adenylyl cyclase stimulators on PGE_2/LTB_4 release in control and COPD-AM. An increase in cellular cAMP-levels in AM is generally associated with de-activation of these cells. Release of cytokines, lysosomal enzymes, plasminogen activator and reactive oxygen radicals is reduced in response to adenylyl cyclase stimulating compounds like isoprenaline, histamine, PGE_2 and PDE-inhibitors [6]. Similarly, phagocytosis and tumoricidal activity are impaired by cAMP- enhancing agents [32]. We were therefore surprised to notice in both AM populations, that enhancement of cellular cAMP-levels by salbutamol and IBMX induced a decrease in PGE_2-release and a rise in LTB_4- production. LTB_4 has potent pro-inflammatory properties [3], sothat this effect of cellular cAMP- enhancing compounds can be regarded as activation of AM. The same holds for the reduced PGE_2-release, which will, via a diminished feed-back signal, result in lower intracellular cAMP-level of the AM themselves and thus enhance their activity.

In control AM, enhancement of intracellular cAMP -level dose-dependently impaired PGE_2-release. Because of low basal PGE_2-release in COPD-AM and the efficiency of the PDE-inhibitor IBMX (1 mM) to inhibit this release, we were not able to obtain in COPD-AM such a good correlation between cellular cAMP-level and PGE_2-secretion as in control-AM. Decreasing the IBMX concentration below 1 mM would theoretically improve this correlation, but this was not possible as such concentrations do not sufficiently protect the hydrolysis of the generated cyclic nucleotides. Apparently, salbutamol modulated cAMP-level and eicosanoid-release via a ß- adrenoceptor mediated mechanism, as both effects could be fully blocked by propranolol.

Interestingly, in contrast to PGE_2-secretion, the LTB_4-release was in both AM populations not clearcut dose-dependent on cAMP, as poor correlations were obtained when changes in LTB_4- release were plotted against the cAMP-content. Still propranolol completely antagonized the effect of salbutamol on LTB_4-release. We therefore conclude, that LTB_4-release is controlled by intracellular cAMP-levels in an indirect way. The question that may be addressed is which other intracellular process cooperates with cAMP-control in modulating the LTB_4-release from AM. In accordance with our previous findings [12], we hypothesize, that impaired metabolism

of arachidonic acid into PGE_2 by adenylyl cyclase stimulants results in a higher availability of the common substrate (free arachidonic acid) for leukotriene synthesis by 5-lipoxygenase. Indeed, the results depicted in figure 10.2., which show a relationship between the decrease in PGE_2- and the increase LTB_4- release, support this hypothesis. It should be noted, that 5- lipoxygenase activation does not exclusively generates LTB_4 but also peptido- leukotrienes (LTC_4, LTD_4 and LTE_4). AM however metabolize arachidonic acid via 5- lipoxygenase, into mainly LTB_4 [5,6,13,27] so that we confined our determinations to this metabolite.

In both control AM and COPD-AM enhancement of the cytosolic levels of cGMP by sodium nitroprusside did not change the production of either PGE_2 or LTB_4. These results suggest, that the cytosolic pool of cGMP, which is stimulated by SNP, is not involved in the regulation of eicosanoid release from alveolar macrophages. This does however not rule out that another cGMP-pool, possibly generated via receptor-mediated mechanism(s), could be physiologically relevant in the control of eicosanoid release. Enrichment of such a secondairy and receptor- mediated cGMP-pool is presumably attained after exposure of macrophages to zymosan, somatostatin or low concentrations of PGE_2 [15,33,38].

Xanthine- derivatives and $ß_2$-adrenoceptor agonists are useful drugs in the therapy of asthma and COPD, though they are mainly effective in the acute relief of bronchoconstriction through their direct dilating effect on bronchial smooth muscle. Present results show, that both salbutamol and IBMX induce the release of the potent chemotactic agent LTB_4 from AM. Hence, these drugs would contribute to the induction of an inflammatory process which is regarded as a typical feature of the late phase response. Indeed, adverse effects of ß- adrenoceptor agonists in the treatment of asthma have been reported. Bronchial responsiveness in children and adults was increased following administration of $ß_2$- adrenoceptor agonists [22,31,41]. Such adverse effects of theophylline- like drugs are scarcely observed, probably because these drugs are administered by different routes and require high concentrations to cause adverse effects. The present results with IBMX suggest however, that theophylline-like drugs (at high concentrations) may indirectly stimulate LTB_4- release from AM through their PDE- inhibitory properties.

In conclusion, enhancement of cAMP- levels of alveolar macrophages by salbutamol and theophylline- derivatives induce a direct decrease in PGE_2- release and an indirect stimulation of LTB_4- release which may contribute to the induction or worsening of the inflammatory processes, associated with COPD and asthma.

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chapter eleven

correlation between basal cyclic amp-levels and the spontaneous release of eicosanoids from human alveolar macrophages. a comparison between controls and patients with chronic obstructive pulmonary disease *

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11.1. summary

The putative correlation between basal cAMP-levels and spontaneous release of eicosanoids was evaluated in different human alveolar macrophage (AM) populations. AM from non-smoking controls (CTRL-AM) displayed some 2.76 pmol cAMP/10^6 cells whereas in smoking CTRL-AM this level was significantly reduced to 75 % (1.58 pmol/10^6 AM). This cAMP-decreasing effect of smoking could also be determined in AM from patients with chronic obstructive pulmonary disease (COPD-AM). There was a significant difference in basal levels of cAMP when corresponding AM were compared. Thus, basal cAMP-levels in COPD-AM were enhanced when compared to CTRL-AM (smokers CTRL-AM vs. smokers COPD-AM as well as non-smokers CTRL-AM vs. non-smokers COPD-AM). A marked difference was determined in AM from smoking COPD patients with bronchial carcinoma (CAR-AM), which showed lowest basal cAMP-levels of all AM-populations studied (34 % of basal cAMP-levels from smoking COPD-AM). These decreased cAMP-levels in CAR-AM coincided with diminished spontaneous release of Prostaglandin E_2 (PGE_2) and Leukotriene B_4 (LTB_4) when compared to CTRL-AM. In addition, incubation with opsonized zymosan

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induced an increase in the release of PGE_2 and LTB_4 from both AM-populations (more pronounced in CTRL-AM) which appeared not to be mediated via cyclic nucleotide-dependent mechanisms. These data indicate that modulation of basal intracellular cAMP-levels by smoking and the presence of pulmonary disorders like COPD and bronchial carcinoma affect the release of eicosanoids from human AM.

Key words: human alveolar macrophages, cyclic AMP, cyclic GMP, prostaglandin E_2, leukotriene B_4, chronic obstructive pulmonary disease (COPD), bronchial carcinoma.

11.2. introduction

Alveolar macrophages (AM) exert multiple actions in order to maintain the pulmonary compartment free of noxious and pathogenic materials. As such, these cells, which are abundantly present in the lung, possess phagocytic and microbicidal activity. In addition AM may mobilize and activate other immune cells (e.g. eosinophils, neutrophils and lymphocytes) to participate in the cascade of inflammatory and immunological pulmonary reactions [2,26]. Coordination of these complicated immune-reactions is attained by the release of numerous biologically active mediators. These mediators interact with extracellular receptors coupled to several effector enzymes to modulate in a complex way many cellular responses (reviewed by Barnes *et al.*) [4]. The second messenger cyclic AMP (cAMP) plays an important part in these intracellular phenomena, as we recently showed that inducers of cAMP-production affect eicosanoid secretion from human AM. Thus, the $ß_2$-selective adrenergic agonist salbutamol and the theophylline-like drug IBMX (both stimulators of cAMP-production) induce decreased PGE_2-release and increased LTB_4-release from human AM [5]. The role of cGMP in the modulation of cellular activity is still unclear.

LTB_4 and PGE_2 possess immunomodulatory potencies for various pulmonary cells including AM themselves [19,25]: LTB_4 is highly chemotactic for several cells known to be involved in the pathogenesis of inflammatory lung disorders like eosinophils and neutrophils [13,17,24]. Prostaglandin E_2 has on the other hand mainly immunosuppressive actions [7,29].

It was previously shown that AM release prostaglandins, thromboxane and also lipoxygenase products when stimulated with zymosan or calcium-ionophore [9,10,11,23]. Interestingly, a number of studies have shown impaired prostanoid secretion in AM

from asthmatic subjects, either basal or upon stimulation with zymosan [12,15]. Increased generation of lipoxygenase metabolites of LTB_4 has been observed in AM from asthmatics [12] and asbestosis patients [14]. In addition, it has been shown that smoking considerably impairs the basal or stimulated release of arachidonic acid metabolites PGE_2, Thromboxane B_2 (TxB_2), LTB_4 and 5-HETE [14,21,22,30].
As basal intracellular cAMP-levels appear to represent an important factor for AM activity, we determined the putative correlation between basal cAMP-levels and PGE_2- and LTB_4-release in different human AM populations, either unstimulated or zymosan-stimulated. In addition, cGMP-levels were determined to investigate whether this second messenger is implicated in eicosanoid production from human AM.

11.3. methods

11.3.1. subjects

Thirty healthy volunteers (female, mean age 31, range from 25 to 40 yrs) were included in the study. Ten subjects were life-long non-smokers whereas 20 subjects were tobacco smokers (> 5 pack years). None of the subjects received any medication prior to the study or had any history of pulmonary disorders. Informed consent for bronchoalveolar lavage (BAL) was obtained. BAL was performed under general aneasthesia with a fiberoptic brochoscope in which four volumes of 50 ml sterile saline were instilled into a subsegmental bronchus of the right middle lobe, followed by gentle aspiration. The obtained BAL fluids were subsequently kept on ice.

11.3.2. COPD patients

In vivo BAL
Eleven patients (5 male, 6 female, mean age 51 years, range from 36 to 66) were included in the study. All of them were diagnosed for COPD according to the criteria of the American Thoracic Society [1] and by history of pulmonary symptoms, chest X-ray film and pulmonary function tests. Mean FEV_1 was 69 ± 6 % and mean FVC was 86 ± 4 % (percentage of the predicted value). Three patients were non smokers whereas 8 patients were tobacco smokers (> 10 pack years). None of the patients included in this study received any medication related to their pulmonary disorders for at least two months prior to the study. The procedure for BAL corresponded with

the protocol described under 'subjects'.

In vitro BAL.
Besides *in vivo* BAL from the above described COPD patients, an *in vitro* lavage procedure was carried out using lung tissues from bronchial carcinoma patients who had undergone lobectomy. According to the criteria of the American Thoracic Society [1], all of these patients were diagnosed for COPD with mean FEV_1 of 66 ± 7 % and mean FVC of 85 ± 8 % (percentage of the predicted value). All of them were tobacco smokers (> 10 pack years). Within 30 min after surgical resection, the tissue was immersed in ice cold Krebs-Henseleit buffer (pH 7.6). AM (referred to as CAR-AM) were recovered by *in vitro* lavage of peripheral airways with the same buffer using a 20 ml syringe.

11.3.3. isolation of alveolar macrophages

BAL-fluids were filtered through surgical gauze and centrifuged at 400 x g (10 min, 4° C). After washing, the pellet was resuspended in Gey Balanced Salt Solution (GBSS) and AM were purified by density gradient centrifugation (400 x g, 30 min, 4° C) using Ficoll-Isopaque (Nycomed, Oslo, Norway) and the resultant AM layer was washed twice. After this step more than 95 % of the cell-suspension consisted of AM as judged by May Grünwald Giemsa staining of cytofuge preparations. The viability of the cells was assessed by dye exclusion test using Trypan blue; AM suspensions with a viability exceding 95 % were used for the experiments.

11.3.4. incubation procedure, eicosanoid- and cyclic nucleotide assays

One ml samples of AM ($2x10^6$/ml) were incubated in GBSS buffer at 37° C in the presence of 1 mM IBMX (3-isobutyl-1-methylxanthine, Janssen Chimica, Beerse, Belgium) or human serum opsonized zymosan (0.5 mg/ml) and 15 or 60 min later AM were spinned down after which the cell-free supernatant (SN) was removed and stored at -80° C for further analysis. Prostaglandin E_2 and LTB_4 liberated in the SN were assayed with commercially available ELISA-kits (Cayman Chemical, Ann Arbor, USA). Detection limits for PGE_2 and LTB_4 were 3 and 1 pg/ml respectively. The cellpellet was resuspended in 150 µl Tris-HCl buffer (pH 7.4), boiled for 3 min and in

the cell-free SN the content of cAMP was determined by radioimmunoassay using [^{3}H] cAMP (Amersham, Amersham, UK) and a high-affinity binding protein as described previously [8]. Cellular cGMP-levels were determined by RIA-assay as recently described [5].

11.3.5. statistical analysis

Data are expressed as means ± standard error of the mean (S.E.M.). Statistical significance was evaluated by the Mann-Whitney U test. A p-value < 0.05 was considered significant.

11.4. results

Intracellular cAMP-levels were determined in the presence of IBMX to protect the produced cAMP to be hydrolysed by phosphodiesterases. Among the different unstimulated AM populations striking differences in cellular cAMP-levels were observed (cf. Table 11.1.).

Table 11.1. Basal cAMP-levels in AM from smoking and non-smoking control subjects, bronchial carcinoma and COPD patients, and COPD-patients.

		cAMP (pmol/10^6 cells)
CTRL-AM	non smokers	2.76 ± 0.14
	smokers	1.58 ± 0.19*
CAR-AM	smokers	0.87 ± 0.12†
COPD-AM	non smokers	3.67 ± 0.14‡
	smokers	2.53 ± 0.19*†

Abbreviations: CTRL-AM, control AM; CAR-AM, AM from bronchial carcinoma patients; COPD-AM, AM from COPD patients; Cyclic AMP-levels were determined following a 15 min incubation period in the presence of 1 mM IBMX. Data are expressed as means ± S.E.M. from 5-20 duplicate experiments. (*): $p<0.05$ as compared to non-smokers within the same population; (†): $p<0.05$ as compared to smoking control subjects; (‡): $p<0.05$ as compared to non-smoking control subjects.

Lowest basal cAMP-levels were found in AM obtained from bronchial carcinoma patients (CAR-AM) whereas highest cAMP-levels were observed in AM obtained from non-smoking COPD-patients (some three fold difference). Within both control-and

COPD-groups, smoking behaviour appeared to have induced a remarkable decrease in basal cAMP-levels (a 75% and 45% reduction respectively). In addition, the manifestation of COPD in both non-smokers and smokers coincided with an enhancement of basal cAMP-levels. A remarkable difference could be observed when AM from smoking bronchial carcinoma patients, diagnosed for COPD (CAR-AM) were compared to AM from smoking COPD patients: although these patients share a common history of pulmonary disorders and smoking habits, basal cAMP-levels were significantly reduced (three-fold) in the former group as compared to AM from smoking COPD patients (Table 11.1.). The same holds when these CAR-AM were compared to AM from smoking controls as a two-fold decrease in cAMP -levels could be observed in CAR-AM.

Table 11.2. Cyclic AMP- and cyclic GMP-concentrations and prostaglandin E_2 and leukotriene B_4-release of AM from smoking control subjects and smoking bronchial carcinoma COPD patients.

stimulus/agent	cAMP (pmol/10^6 cells)	cGMP (fmol/10^6 cells)	PGE_2 (ng/10^6 cells)	LTB_4 (ng/10^6 cells)
unstimulated				
CTRL-AM	1.36 ± 0.14	8.41 ± 1.13	120 ± 5	19.2 ± 4.2
CAR-AM	0.95 ± 0.10*	9.06 ± 1.33	49 ± 11*	14.8 ± 0.5
+ IBMX (1 mM)				
CTRL-AM	2.70 ± 0.29†	12.96 ± 1.69†	90 ± 9†	24.2 ± 4.5
CAR-AM	1.47 ± 0.17*‡	15.96 ± 1.93‡	11 ± 2*‡	18.3 ± 2.0
zymosan stimulated				
CTRL-AM	1.03 ± 0.13	7.54 ± 1.54	137 ± 10†	34.9 ± 6.8†
CAR-AM	1.19 ± 0.04	8.81 ± 0.81	64 ± 4*	17.2 ± 0.2*‡

Abbreviations: CTRL-AM, control AM; CAR-AM, AM from bronchial carcinoma patients; AM were incubated for 60 min in the absence or presence of 1 mM IBMX, with or without opsonized zymosan (0.5 mg/ml). Data are expressed as means ± S.E.M. from 13-18 duplicate experiments. *: $p<0.02$ as compared to CTRL-AM within the same (un) stimulated group; †: $p<0.02$ as compared to unstimulated CTRL-AM; ‡: $p<0.02$ as compared to unstimulated CAR-AM.

To enable the measurement of spontaneous PGE_2-and LTB_4-release from non-stimulated and zymosan-stimulated AM-populations the incubation time was subsequently prolonged to one hour. Due to a) low availability of COPD-AM and b) low number of AM in BAL fluids of non-smoking individuals [21,22,30], we were not able to determine the eicosanoid release by these AM-populations. In analogy with basal cAMP-data determined after 15 min, incubation of these cells for one hour in the

presence of IBMX induced an increase of basal intracellular cAMP-levels of some 55% in both CTRL-AM and CAR-AM (cf. Table 11.2.). One hour after incubation, CAR-AM contained a 2-fold lower cAMP-level than smoking control AM which, in both unstimulated and IBMX-incubated coincided with impaired spontaneous PGE_2-release (minus 60% and 88% respectively, compared to CTRL-AM). The LTB_4-release was decreased by 23% and 24% respectively compared to CTRL-AM though not to a significant level.
Simultaneously, in all AM populations, basal cGMP-levels were comparable and enhanced to the same extend (approx. 60%) after incubation with IBMX.
The phagocytic stimulant opsonized zymosan did not affect intracellular content of cAMP or cGMP in CTRL-AM or CAR-AM, but induced an increase in the release of eicosanoids. The release of PGE_2 was enhanced with 14% and 31% whereas the LTB_4-release was increased by 82% and 16% in CTRL-AM and CAR-AM respectively.

11.5. discussion

In the present report, we have shown that basal eicosanoid release from AM is strongly associated with intracellular cAMP-levels. As leukotrienes do not affect cAMP-levels via direct and receptor-mediated adenylyl cyclase stimulation, we propose that basal PGE_2-release provides a feedback signal to modulate cAMP-levels in these cells: low concentrations coincide with low basal cAMP-levels, and high PGE_2-concentrations are reflected with high basal cAMP-levels.
Diminished basal cAMP-levels as observed in smokers AM from both controls ans COPD patients could well be ascribed to this phenomenon since smoking has been shown to decrease the capacity of AM to metabolize and release prostanoids and leukotrienes. However, smoking affects both metabolic pathways of arachidonic acid (and does not induce a shift towards either lipoxygenase or cyclooxygenase activity) it probably induces a decreased PLA_2 activity or diminished availability of arachidonic acid for further metabolic conversion.
We recently described that the $ß_2$-selective adrenergic agonist salbutamol and the theophylline-like compound isobutyl-methyl-xanthine (IBMX) affected eicosanoid secretion from human AM via stimulation of adenylyl cyclase and the subsequent enhancement of intracellular cAMP-levels. Thus, PGE_2-and LTB_4 release were decreased respectively augmented by these drugs frequently used in the treatment of asthma and COPD [5]. Indeed, incubation of CTRL-AM or CAR-AM with IBMX

resulted in increased cAMP-levels and in reduced PGE_2-release, the latter being more pronounced in CAR-AM. As 1) we were only able to determine eicosanoid-release from smokers AM and 2) smoking may have affected this release (as described above), the expected shift towards lipoxygenase activity was not apparent in CTRL-AM. In CAR-AM however, this shift was more pronounced, probably due to to the profound decreasing effect of IBMX on PGE_2-release in these cells. Indeed, AM from COPD and asbestosis patients have been shown to release more lipoxygenase products as compared to AM from controls [11,14].

Using guinea pig AM, we have previously shown that an enhanced adenylyl cyclase responsiveness in sensitized and antigen challenged AM possibly derives from an increment of α_s-subunits of the guanine nucleotide binding proteins [6]. The present data using AM from COPD-patients suggest that the development of COPD induces in human AM a similar response. Thus, AM from COPD-patients, in analogy with sensitized guinea pigs, may have been primed *in vivo* which results in enhanced basal cAMP-levels. As indicated above, the enhanced adenylyl cyclase responsiveness results in altered eicosanoid release from these cells. Further analysis of adenylyl cyclase responsiveness to stimulatory agents and studies using membrane fractions of AM from COPD patients, would enable to establish whether a similar phenomenon has indeed been induced in COPD.

AM obtained from bronchial carcinoma patients (CAR-AM) contained lowest basal cAMP-levels among all AM populations analyzed. In addition to bronchial carcinoma, these patients were also diagnosed for COPD. One might therefore assume that these CAR-AM would contain similar basal cAMP-levels as AM from smoking COPD patients and not a threefold lower level. Apparently, the local inflammatory and carcinomatous environment greatly affects the behaviour of this AM population. Moreover, smoking history, local inflammation and the presence of carcinomas seem to cooperate in the down-regulation of basal cAMP-levels of AM and subsequently in the modulation of the activity of this cell.

In accordance with previous studies [16,26], the phagocytic stimulant zymosan enhanced in control AM and CAR-AM the release of PGE_2 but mainly LTB_4, (the increase in PGE_2-release from CTRL-AM was marginal whereas in CAR-AM this PGE_2-release was not statistically significant from unstimulated AM). This enhancement of eicosanoid release by zymosan appeared not to be mediated by modulation of intracellular cyclic nucleotide levels (cAMP or cGMP) as was recently shown for salbutamol or IBMX [5]. Using these drugs, enhancement of cAMP-levels led to a

decreased PGE_2-and an increased LTB_4-release from human AM. Futhermore, it was previously shown that zymosan induces the production and release of both cyclooxygenase and lipoxygenase metabolites [3,20,27] suggesting that phagocytosis triggers arachidonic acid metabolism via mechanisms which are probably not directly linked to production of second messengers but rather acts as a non-specific stimulus to affect arachidonic acid metabolism. Indeed, during phagocytosis, conformational changes of the membrane have been shown which could induce the liberation of arachidonic acid and its subsequent conversion via cyclooxygenase and lipoxygenase pathways [18].

The role of cGMP in eicosanoid production from AM remains unclear. As we have recently shown, cGMP-levels in AM were stromngly enhanced by sodium nitroprusside which was however not reflected with altered eicosanoid release [5]. The present results, showing no difference in cGMP-levels between CTRL-AM and CAR-AM while PGE_2- and LTB_4-release showed clear differences, substantiate these findings indicating that alterations in intracellular cGMP-levels do not affect eicosanoid production in AM.

Taken together, our data suggest that basal cAMP-levels in AM are strongly associated with spontaneous production of eicosanoids. External factors like smoking, drugs and bronchial carcinoma may cooparate to affect cAMP- and eicosanoid production of AM and therefore affect the modulatory function of this celltype in pulmonary inflammation.

11.6. references

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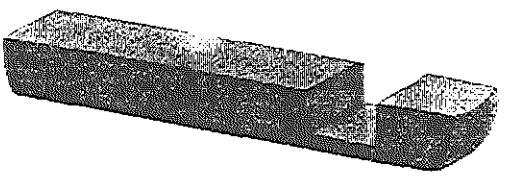
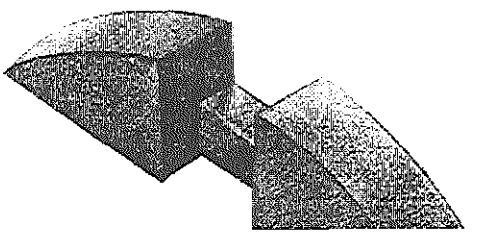
PART FIVE

GENERAL DISCUSSION AND SUMMARY





chapter twelve
general discussion

During the past decade, drug therapy of asthma and COPD has clearly shifted from relief of typical clinical manifested symtoms (cough, wheezing and airway obstruction all resulting from bronchoconstriction) to a more causal therapy aimed at the reduction of symptoms resulting from pulmonary inflammation which is undoubtedly associated with the pathogenesis of both diseases. Indeed, anti-inflammatory drugs (like corticosteroids and drugs preventing mediator-release) are succesfully applied in the treatment of asthma and COPD. Though corticosteroids may reduce many different aspects of pulmonary inflammation [8], they will never completely abolish it, indicating that pulmonary inflammation is not only difficult to treat but also constitutes an extremely complex feature of asthma and COPD. This complexity (and its subsequent difficult management) arises from the involvement of an array of diverse components and closely interrelated actions. Gathering more information on the individual components (like AM in this study) is one way to tackle the complexity of the problem.

Modulators of cellular activity, like drugs and mediators, affect the levels of intracellular second messengers via interactions with different transmembrane signalling systems. Disease may modify the conformation of these signalling systems [2]. Therefore, besides measuring the production and release of mediators, the study of their effects and generation of intracellular messengers should also include putative modification of their signal transfer. More specifically, the transmembrane signalling system of adenylyl cyclase in alveolar macrophages (AM) and its conformational alterations by intrinsic factors (sensitization or the disease state of COPD and asthma itself) and external stimuli like mediators, drugs and smoking was the topic of the present study.

Using the guinea pig model for human allergic asthma we have shown that conformational changes in the transmembrane signalling system of adenylyl cyclase (AC) indeed occur. The responsiveness of AC to stimulatory agents was enhanced in sensitized and antigen challenged AM probably due to an increased number of α_s-subunits of the coupling G proteins. Alternative explanations for the enhanced responsiveness of AC like an imbalance of G_s/G_i-status, increased receptor number or affinity or enhanced activity of the catalytic moiety could be excluded. These findings indicate that sensitization renders AM more susceptible to inflammatory

mediators like PGE_2 and histamine and ß-adrenergic agonists to modulate functional activity. Considering that increased cAMP leads to diminished phagocytosis, enzyme secretion and oxygen radical production (i.e. primary functions), sensitization (or activation of the immune system) primes AM resulting in a down-regulation of primary functions but not of secondary functions like mobilization of immune cells (see below).
It remains difficult to extrapolate results from an animal model to human conditions, especially within the complex pathophysiology of asthma. The ovalbumine-sensitized guinea pig model is generally regarded as a model for human allergic asthma as it exhibits similar typical characteristics like physiological responses to antigenic challenge and hyperreactivity to non-specific stimuli. Sensitization with relative high doses of ovalbumin (milligramms) induces not only IgG-antibody formation but also the production of IgE-antibodies, indicating once again that this model is reasonably suitable as a model for human allergic asthma. Since we were unable to obtain BAL-fluids from *allergic* asthmatics (because of poor availablity and ethical problems), the interpretation of the results obtained from the guinea pig model remains difficult. Circumstantial evidence suggests that some features of AM from allergic asthmatics are indeed down-regulated, reflecting some similarity with the guinea pig model [7]. One should keep in mind that, besides species differences, differences in isolation- and incubation procedures render the comparison even more difficult.
In contrast to guinea pig AM, no evidence was found for enhanced AC responsiveness in human *non allergic* AM. Basal cAMP levels were clearly increased in AM from both COPD patients and asthmatics but the AC responsiveness to stimulatory agents was severely decreased (and not increased as in guinea pig AM). Both effects cannot be explained by assuming a similar conformational change in the membrane transduction system of AC as observed in AM from sensitized and antigen challenged guinea pigs. A plausible reason for the enhanced basal cAMP levels in human AM would be that the local inflammatory environment generated a variety of mediators which induced enhanced cAMP production of these cells *in vivo*. For the same reason, AM from asthmatics and COPD patients may have become less sensitive to these AC stimulatory agents since continuous exposure of receptors to stimulatory agents is known to induce heterologous desensitization ultimately resulting in diminished AC responsiveness.

In *summary*, immunological challenge of guinea pigs provides a priming signal for AM

inducing conformational changes of the AC transmembrane signalling system (more specifically the α_s-subunits) which result in increased basal cAMP levels and increased AC responsiveness rendering these cells more susceptable to down-regulation of primary effector functions. Increased basal cAMP levels in human AM from asthmatics and COPD patients do not derive from similar changes in the AC-system but probably result from interactions of mediators with the AC system which consequently induce desensitization and diminished AC responsiveness. At this moment it is not certain whether these differences between the guinea pig model and human conditions arise from the absence or presence of a certain priming signal. Future studies should therefore include research on:

- the role of immunological processes in the pathophysiology of asthma and COPD i.e. studying the contribution of T- and B-lymphocytes to pulmonary inflammation;
- the funtion of interleukins as putative priming signals;
- the responsiveness of AM and its progenitors (monocytes) to cytokines.

The outcome of these studies should establish the role of immunological processes in pulmonary inflammation and whether the guinea pig model can be considered an appropriate model for human allergic asthma. Based on our data, it can be concluded that the guinea pig model is not suitable as a model for chronic obstructive pulmonary disease or non-allergic asthma.

External stimulation of cAMP production does not lead to a general down-regulation of macrophageal activity. We have demonstrated that increased production (or diminished breakdown) of cAMP results directly in decreased PGE_2-production and indirectly in enhanced LTB_4-production. Extrapolating these findings to the AC responsiveness of AM suggests that AM from COPD patients and asthmatics are less susceptible to putative adverse effects of AC stimulatory agents (LTB_4-secretion) than AM from controls. Beta-adrenergic agonists and theophylline-like derivatives are succesfully applied in the treatment of asthma and COPD. Their beneficial effects, which are reflected with a direct relief of bronchoconstriction, are confined to bronchial smooth muscle (relaxation via cAMP enhancement). A(nother) side-effect of (long-term) treatment with ß-adrenergic agonists and theophylline-like drugs is, as described above, the production of leukotrienes (predominantly LTB_4 from AM), which

may contribute to the worsening of symptoms despite a decreased AC responsiveness in AM from COPD patients and asthmatics compared to controls. However, both types of drugs appear to be beneficial in the treatment of asthma and COPD suggesting that their efficacy, which is aimed at relief (not cure) of symtoms, is therapeutically more relevant than their adverse immunostimulatory action. For the same reason, one may question the development and use of long-lasting ß-sympaticomimetics.

In macrophages, the balance between arachidonic acid metabolism via cyclooxygenase and lipoxygenase pathways is generally considered to be involved in the control cellular activity. Under normal steady state conditions, resident or 'inactive' macrophages exert relatively high production of cyclooxygenase metabolites and low lipoxygenase activity (in AM predominantly PGE_2 and LTB_4 respectively). Prostaglandin E_2 interacts with AC (via feedback mechanisms) to stimulate AC resulting in relatively high levels of intracellular cAMP concentrations (cf. Figure 12.1., panel A). In 'activated' (elicited) macrophages, arachidonic acid metabolism is shifted towards the lipoxygenase pathway resulting in decreased production of cyclooxygenase metabolites and subsequently in a diminished feedback signal leading to a decrease in intracellular cAMP concentration (panel B). Alveolar macrophages are, due to their location in the pulmonary compartment, continuously exposed to putative harmful influences indicating that AM may be classified as 'activated' macrophages. This assumption is substantiated when basal cAMP levels of AM are compared (under the same conditions) to peritoneal macrophages (PM) from the same species. Thus, basal cAMP levels in AM are substantially lower when compared to basal cAMP levels in resident PM [1]. In addition, when arachidonic acid metabolism of AM and PM within different species are compared, PM indeed produce more cyclooxygenase and less lipoxygenase metabolites than AM [5,6,9].
As smoking decreases basal intracellular cAMP levels in AM (cf. panel C), it is tempting to speculate that this may result from a diminished feedback signal caused by decreased production of cyclooxygenase metabolites. Smoking has however been shown to diminish the production of both cycloxygenase and lipoxygenase metabolites [4] indicating that smoking affects arachidonic acid metabolism in a previous stage of the metabolic routes to eicosanoid formation, possibly by decreasing phospholipase A_2 activity or decreasing the availability of free arachidonic acid.

Basal cAMP levels and basal PGE_2-production in AM from COPD patients who, in addition to COPD were also diagnosed for bronchial carcinoma, were significantly reduced compared to smoking controls whereas LTB_4-production remained unaltered (panel D). Due to the chronic inflammatory and carcinogenic environment, AM may well be continuously activated as these cells exhibit low cAMP levels and low cyclooxygenase metabolism. A relatively high lipoxygenase activity in these CAR-AM was not observed probably caused by 1) the additional effect of smoking (decreasing both cyclooxygenase and lipoxygenase pathways) and 2) tumoricidal activity of macrophages is predominantly associated with LTC_4-production [3] and we only measured LTB_4 production.

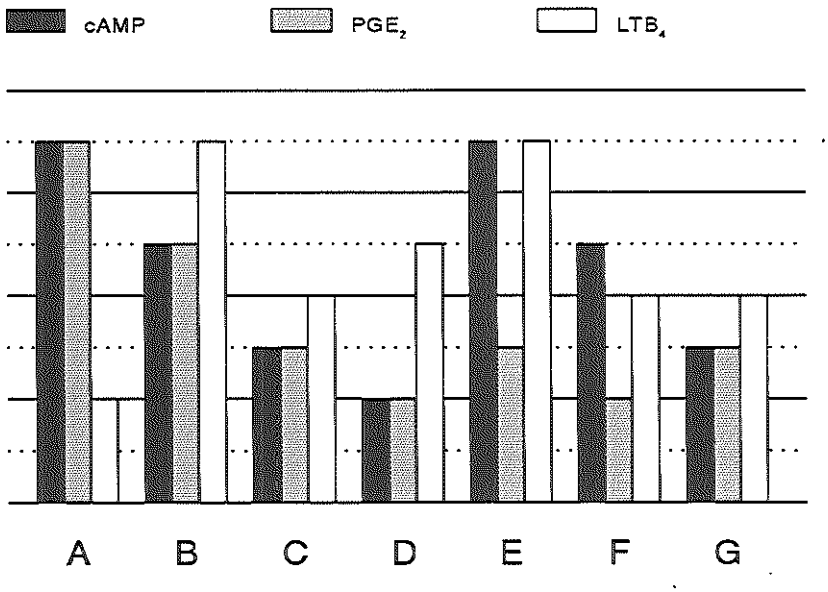


Figure 12.1. Correlations between basal cAMP-, PGE_2- and LTB_4-concentrations in (alveolar) macrophages. For abbreviations see text.

In AM from COPD patients without bronchial carcinoma (COPD-AM), basal cAMP levels were increased (not decreased) compared to controls (cf. panels E and F). In addition, the AC responsiveness to stimulatory agents was diminished (desensitization). As outlined in chapter nine, both effects are interrelated, resulting from interactions of stimulatory agents with the AC transmembrane signalling system.

It seems therefore likely that increased basal cAMP levels in COPD-AM resulted from persistent AC stimulation. External stimulation of cAMP however, results in decreased PGE_2-production and (indirectly) increased LTB_4-production, suggesting that although CAR-AM and COPD-AM differ in basal cAMP levels, a quite similar arachidonic acid metabolism may be observed. This difference in basal cAMP levels is somewhat diminished when CAR-AM are compared to smoking COPD-AM (panel F).
Finally, when basal cAMP levels in AM from smoking controls and asthmatics were compared, no differences were observed (panel G). Although we obtained data from only two (non allergic) asthmatics, literature on arachidonic acid metabolism (showing no difference between controls and asthmatics) indeed substantiates our findings.

In *summary*, 'activation' of AM by agents or events, probably via Ca^{2+}-dependent mechanisms, is reflected by a shifted balance of arachidonic acid metabolism towards increased lipoxygenase activity (and obviously a decreased cyclooxygenase activity leading to a diminished feedback signal to decrease intracellular cAMP concentrations). External stimulation of AC, thus enhanced cAMP concentrations, decreases cyclooxygenase activity to promote lipoxygenase activity resulting in a similar disturbance of the balance of arachidonic acid metabolism. The latter mechanism does not lead to diminished cAMP concentrations as the exogenous stimulus affects the same membrane transduction signal (AC) as endogenous PGE_2 thus masking its feedback signal. Hence, when basal cAMP levels are applied as parameters for 'state of activation' for AM (and macrophages in general), the study should include the AC responsiveness and clearly state the origin of the macrophages.

A physiological example of such an imbalance of arachidonic acid metabolism and its involvement in cellular activity is illustrated by the actions of PAF on AM. At low concentrations, this lipid mediator preferentially stimulates cycloxygenase activity to produce prostaglandins which, via feedback increase cAMP levels. At higher concentrations, lipoxygenase activity is enhanced which, as described above, results in decreased prostaglandin production, diminished feedback signal and decreased cAMP levels. PAF probably exerts its actions on AM via receptor-stimulation of phospholipase C which induces the elevation of IP_3 and DAG concentrations. Both second messengers promote arachidonic acid metabolism via distinct mechanisms.

IP_3 enhances intracellular Ca^{2+}-concentrations which incraeses phospholipase A_2 activity (which in turn results in arachidonic acid liberation) whereas DAG can be converted into arachidonic acid via lipases. In addition, increased concentrations of intracellular Ca^{2+} preferentially stimulate lipoxygenase activity which, in contrast to cyclooxygenase, is a calcium-dependent enzyme.
Under normal physiological conditions (compared with naive AM), only minor levels of PAF are circulating but in response to specific allergens (anaphylactic reactions, compared with antigen challenged AM), substantantial amounts of PAF can be generated from a variety of pulmonary cells which may induce the production of lipoxygenase metabolites (which are either chemotactic or bronchoconstrictive). In this way, it may be explained why PAF is such a potent pro-inflammatory mediator in asthma.

Though PAF may account for many of the pathological features observed in asthma and COPD it remains only one small piece in the complex puzzle of pulmonary inflammation. The same holds for leukotrienes, cytokines and other mediators, once again emphasizing that not one specific mediator is responsible for inducing pulmonary inflammation.

One should keep in mind that present or near-future treatment of pulmonary inflammation is or will be based on present knowledge which is still limited or at least not sufficient. We have confined our study to the alveolar macrophage which certainly plays a predominant role in the modulation of pulmonary inflammation but remains a small piece of the complex puzzle of pulmonary inflammation. To manage and treat pulmonary inflammation, the full puzzle needs to be solved; this requires more insight into several unexplored mechanisms which may all contribute to the pathophysiology of asthma and COPD:

- The role of T-and B-lymphocytes and their products in pulmonary inflammation in general and allergic asthma in particular.
- The role of epithelial damage and neurogenic inflammation i.e. the effects of substance P, vasoactive intestinal peptide (VIP) and other neuropeptides on the different components of the pulmonary compartment.
- The synergistic or antagonistic actions of pulmonary cells i.e. the net result of

the effect(s) of inflammatory mediators on a population of various cells (including bronchial smooth muscles).

- The interactions of second messengers to modulate the cellular activity of immune cells.

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chapter thirteen
summary

Part one consists of a general introduction which contains an overview of pulmonary inflammatory reactions in asthma and COPD, the role of alveolar macrophages within this process and the modulation of cellular activity by second messengers.
In **chapter one**, different aspects of pulmonary inflammation are summarized including its association with asthma and COPD, the differentiation of pulmonary cells into primary and secondary effector cells and the function of mediators as modulators and cross-talk factors between cells. Asthma and COPD share many clinical characteristics which makes the classification of pulmonary diseases rather difficult. Histopathological analysis of lung biopsies, mucus secretions or bronchoalveolar lavage (BAL) fluids of both asthmatics and COPD patients shows however that pulmonary inflammation is inevitably involved in the pathogenesis of both diseases. The cascade of multiple reactions including interactions between pulmonary cells and secretion and actions of mediators results in typical inflammation-like features which, from a physiological point of view, may be beneficial (to maintain normal lung homeostasis) but may, under certain conditions, lead to exaggerated and uncontrollable responses.
To understand the mechanisms of pulmonary inflammation, information on the involved pulmonary cells (their reactivity, ability to respond to external stimuli) and mediators (source, secretion, breakdown and site of extracellular and intracellular activity) is mandatory. Furthermore, it should be noted that inflammation (including pulmonary inflammation) is not induced by the action of a single cell type or mediator but results from actions of a variety of cell types and mediators.
Chapter two focusses on the role of alveolar macrophage (AM) within the process of pulmonary inflammation, its typical functions and the modulation of these functions in asthma and COPD by external factors like drugs and smoking. Alveolar macrophages may be regarded as prominent cells in pulmonary inflammation. Due to their anatomical location, these cells exhibit a large capacity to respond to and cope with specific and non-specific stimuli by means of phagocytosis-related features. In addition, AM may regulate the activity of other pulmonary cells via production and secretion of an enormous variety of agents like enzymes, arachidonic acid metabolites, platelet activating factor, cytokines and growth factors.
These agents (primary messengers, but also drugs, toxins, hormones, etc.) perform their action via cell-surface receptors coupled to transmembrane signalling systems. These systems translate extracellular information into intracellular signals (second

messengers) thus modulating cellular activity. In **chapter three** the mechanisms of these processes are described in more detail. As cyclic AMP represents one of the most important second messengers involved (emphasized in this chapter) in the control of cellular activity, elucidation of its transmembrane signalling system (adenylyl cyclase), modulation of its concentration by external stimuli and its effect on cellular functions of control and 'affected' AM contribute to a better understanding of the role of this cell in (diseases associated with) pulmonary inflammation. Finally, **chapter four** catagorizes the aims of the present study.

Part two describes the responsiveness of AC in AM to a variety of different stimuli in a guinea pig model for allergic asthma which was chosen to obtain a controlled model to study this transduction signalling system.
Chapter five deals with the effects of sensitization and antigen challenge on the AC responsiveness in AM to the inflammatory mediators PGE_2, PGI_2 and histamine and the ß-adrenergic agonists isoprenaline and salbutamol. The ability of AC to respond to these stimuli was enhanced in sensitized and antigen challenged AM, probably due to alterations in the transmembrane signalling system. Furthermore, it was shown that AM possess functional $ß_2$-adrenergic and H_2-histaminergic receptors coupled to adenylyl cyclase.
In **chapter six**, the suggested alterations at post-receptor levels were confirmed in membrane fractions of the different AM populations as this approach allows to investigate transmembrane signalling systems more specifically (with respect to the different components). Receptor conformation as well as catalytic activity of AC appeared unaltered whereas the coupling capacity between them via G_α subunits (probably increased number of α_s-subunits) was enhanced.

Part three describes the effects of platelet activating factor (PAF) on intracellular arachidonic acid metabolism and the AC-system in AM. As demonstrated in **chapter seven**, PAF interacts with AM through specific receptors which are not directly coupled to AC but via its action on arachidonic acid metabolism. Low PAF-concentrations increase whereas higher PAF-concentrations decrease intracellular levels of cAMP via cyclooxygenase and lipoxygenase dependent pathways respectively. Furthermore, in contrast to control AM, these PAF-effects were observed only in sensitized and antigen challenged AM.
These suggestions were substantiated in **chapter eight** in which it was shown that

low PAF-concentrations indeed enhance cyclooxygenase activity whereas higher PAF-concentrations stimulated lipoxygenase activity (to yield enhanced secretion of PGE_2 and LTB_4 respectively). The PAF-induced increased PGE_2-production observed in naive AM was apparently not sufficient to stimulate AC as cAMP-levels were not enhanced in these cells. Due to conformational changes in transduction signalling (described in chapters five and six) the PAF-induced PGE_2-production in antigen challenged AM appeared to be sufficient to stimulate AC and increase cAMP-levels in these cells. Furthermore, it was postulated that the PAF-induced decrease in cAMP levels (at higher concentrations) results from a metabolic shift towards increased lipoxygenase activity negatively affecting cyclooxygenase activity (thus diminishing PGE_2-production). As PGE_2 provides a feedback signal (to stimulate AC), diminished PGE_2-production results in decreased cAMP levels.

In **part four** studies on basal cAMP levels, AC responsiveness and its association with arachidonic acid metabolism of human AM are summarized.
In analogy with the study presented in chapter five, **chapter nine** decribes the AC responsiveness to inflammatory mediators and ß-adrenergic agonists in human AM obtained from control subjects, asthmatics and COPD patients. With respect to basal levels of cAMP, AM from asthmatics and COPD patients showed higher levels compared to controls (in parallel with sensitized and/or antigen challenged vs. naive guinea pig AM). The responsiveness of AC to different stimuli was however significantly decreased in AM from asthmatics and COPD patients as compared to controls, possibly as a result of (heterologous) desensitization of stimulatory receptors.
In **chapter ten** a more clinical approach was used to evaluate the role of AM in pulmonary inflammation (COPD in particular) by means of studying the effects salbutamol and a theophylline-like drug on arachidonic acid metabolism. Both drugs cause direct smooth muscle relaxation via their cAMP-enhancing effect. In AM however, increases in cAMP-concentrations result in diminished cyclooxygenase activity and consequently in enhanced lipoxygenase activity (already observed in the PAF-induced effects on competitive arachidonic acid metabolism, chapters seven and eight). Enhanced lipoxygenase activity results in increased production and release of LTB_4 which is an extremely potent chemoattractive substance for secondary inflammatory cells. Furthermore, it was shown that alterations in levels of cGMP do not affect arachidonic acid metabolism in AM.

Finally, in **chapter eleven** the effect of smoking on the correlation between basal levels of cAMP and cGMP and spontaneous and zymosan-induced arachidonic acid metabolism in AM from controls and COPD patients was evaluated. Alterations in basal cAMP (not cGMP) levels coincide with differential production of PGE_2 and LTB_4 which is negatively affected by smoking. The zymosan-induced release of arachidonic acid metabolites appeared to involve a mechanism distinct from AC or guanylyl cyclase.

In **deel een** wordt een algemene inleiding gegeven bestaande uit een overzicht van pulmonale ontstekingsreacties betrokken bij astma en chronisch aspecifieke respiratoire aandoeningen (CARA of COPD), de rol van de alveolaire macrofaag (AM) binnen deze processen en de modulatie van de cellulaire activiteit van de AM door zogenaamde tweede boodschappers.

In **hoofdstuk een** worden de verschillende aspecten van pulmonale ontsteking samengevat waaronder de rol ervan binnen astma en CARA, de differentiatie van longcellen in primaire en secundaire effector cellen en de functie van mediatoren als modulatoren van en middelen van communicatie tussen de verschillende celtypen in de long. Omdat het ziektebeeld van astma en CARA op vele klinische aspecten overeenkomen, is de classificatie van patiënten die leiden aan astma of CARA een moeilijke. Histopathologische analyse van longbiopten, slijmsecreties en bronchoalveolaire lavages (BAL) van astmatici en CARA-patiënten geeft aan dat pulmonale ontsteking ontegenzeggelijk verbonden is met beide longziekten. De opeenvolging van vele processen, waaronder de interacties tussen longcellen en de productie en acties van mediatoren, resulteert in typische ontstekings-achtige kenmerken die, vanuit een fysiologisch oogpunt, gunstig kunnen zijn (ter handhaving van normale longhomeostase) maar die onder bepaalde omstandigheden tot een overmatige en oncontroleerbare respons kunnen leiden.

Om de mechanismen die ten grondslag liggen aan pulmonale ontsteking te kunnen begrijpen, is kennis omtrent de betrokken longcellen (reactiviteit, vermogen om op externe stimuli te reageren) en mediatoren (oorsprong, productie, afbraak en plaats van extra- en intracellulaire activiteit) onontbeerlijk. Hierbij dient opgemerkt te worden dat ontsteking (dus ook pulmonale ontsteking) niet veroorzaakt wordt door de actie van één enkel celtype of mediator maar door acties van verschillende cellen en mediatoren.

In **hoofdstuk twee** wordt de rol van de AM binnen het proces van pulmonale ontsteking beschreven, haar typische functies en de modulatie van deze functies in astma en CARA en door externe factoren zoals farmaca en roken. Alveolaire macrofagen kunnen beschouwd worden als belangrijke cellen in pulmonale ontsteking. Als gevolg van hun anatomische locatie, vertonen deze cellen een groot vermogen te reageren op en handelen naar specifieke en niet-specifieke stimuli door middel van fagocytose-gerelateerde functies. Bovendien kunnen AM de activiteit van andere longcellen beïnvloeden door de productie en uitscheiding van een verscheidenheid aan producten zoals enzymen, arachidonzuur metabolieten, plaatjes

activerende factor, cytokinen en groei-factoren.
Deze producten (primaire boodschappers, maar ook farmaca, toxinen, hormonen, etc.) oefenen hun werking uit via receptoren op het cellulaire membraan die gekoppeld zijn aan membraan transductie systemen. Deze systemen vertalen extracellulaire informatie naar intracellulaire signalen (tweede boodschappers) die op hun beurt de cellulaire activiteit kunnen beïnvloeden.
De mechanismen die betrokken zijn bij deze processen worden in **hoofdstuk drie** nader toegelicht. De nadruk wordt in dit hoofdstuk gelegd op een van de belangrijkste tweede boodschappers, het cyclisch AMP (cAMP). Met name wordt hierin besproken uit welke componenten het membraan transductie systeem bestaat dat verantwoordelijk is voor de productie van cAMP (adenylyl cyclase), hoe deze productie via externe factoren beïnvloed kan worden en op welke manier cAMP de cellulaire activiteit van AM kan moduleren.
Tenslotte wordt in **hoofdstuk vier** beschreven hoe onderzoek naar het adenylyl cyclase, cAMP en functionele activiteit van de AM kan bijdragen tot een beter inzicht in de functie van deze cel in (ziekten die geassocieerd zijn met) pulmonale ontsteking.

In **deel twee** worden de effecten van verschillende stimuli op de reactiviteit van het adenylyl cyclase systeem beschreven in AM afkomstig van cavia's. Het beschreven cavia-model werd gebruikt teneinde een gecontroleerd model voor humaan allergisch astma te verkrijgen waarin het membraan transductie systeem onderzocht kon worden.
Hoofdstuk vijf beschrijft de effecten van immunologische sensitizatie en antigeen blootstelling (antigen challenge) op de reactiviteit van het adenylyl cyclase voor de ontstekingsmediatoren prostaglandine E_2 (PGE_2), prostacycline (PGI_2) en histamine en de ß-adrenerge agonisten isoprenaline en salbutamol. Deze reactiviteit voor de verschillende stimuli bleek toegenomen te zijn in gesensibiliseerde en antigen challenged AM, waarschijnlijk het gevolg van veranderingen in het membraan transductie systeem van het adenylyl cyclase. Bovendien werd aangetoond dat AM functionele $ß_2$-adrenerge en H_2-histaminerge receptoren bezitten die gekoppeld zijn aan het adenylyl cyclase.
Deze veronderstelde veranderingen in het membraan transductie systeem werden bevestigd in een studie beschreven in **hoofdstuk zes**. Hierbij werd in membraanfracties van de verschillende AM populaties aangetoond dat er, o.i.v.

sensitizatie of antigen challenge, geen veranderingen zijn opgetreden op receptorniveau (dichtheid en affiniteit) of in de activiteit van het enzym adenylyl cyclase, maar dat de toegenomen reactiviteit van het adenylyl cyclase het gevolg was van een verbeterde koppeling tussen receptor en enzym via G_s-eiwitten (waarschijnlijk door een verhoogd aantal α_s-subunits).

In **deel drie** worden de effecten van de lipide mediator plaatjes activerende factor (PAF) op het arachidonzuur metabolisme en het adenylyl cyclase systeem van de AM beschreven.
In **hoofdstuk zeven** werd aangetoond dat PAF via specifieke receptoren, die niet direct gekoppeld zijn aan het adenylyl cyclase systeem, het cAMP gehalte toch kan beïnvloeden. In lage concentraties bleek PAF het cAMP gehalte te stimuleren en in hoge concentraties te verlagen, een effect dat veroorzaakt werd via stimulering van het cyclooxygenase (lage concentraties) respectievelijk het lipoxygenase (hoge concentraties), beide enzymen betrokken bij het arachidonzuur metabolisme. Daarnaast bleek het bifasisch effect van PAF op het adenylyl cyclase systeem uitsluitend in gesensibiliseerde en antigen challenged (niet in controle) AM aangetoond te kunnen worden.
Deze bevindingen werden in **hoofdstuk acht** nader geanalyseerd middels de bepalingen van de door PAF geïnduceerde concentraties van PGE_2 (een cyclooxygenase product) en leukotriene B_4 (LTB_4, een lipoxygenase product). In lage concentraties bleek PAF inderdaad in staat de PGE_2 productie van AM te stimuleren en in hogere concentraties de productie van LTB_4. In controle AM was de door PAF geïnduceerde PGE_2 productie echter onvoldoende om het adenylyl cyclase systeem te stimuleren waardoor er geen veranderingen in cAMP gehalte waargenomen werden in deze cellen. Dankzij veranderingen in het membraan transductie systeem in antigen challenged AM (beschreven in hoofdstukken vijf en zes) was de door PAF geïnduceerde PGE_2 productie in antigen challenged AM wel voldoende om het cAMP gehalte te verhogen. In hogere concentraties bleek PAF een verschuiving van het arachidonzuur metabolisme te bewerkstelligen resulterend in een verhoogde LTB_4 productie (stimulatie van het lipoxygenase) en een verlaagde PGE_2 productie (remming van het cyclooxygenase). Deze verlaagde PGE_2 productie veroorzaakte een verminderd feedback signaal om het adenylyl cyclase te stimuleren wat uiteindelijk resulteerde in een verlaagd cAMP gehalte.

In **deel vier** worden de resultaten besproken van studies met humane AM betreffende cAMP gehaltes, reactiviteit van het adenylyl cyclase en hun associatie met het arachidonzuur metabolisme.
Analoog aan de studies beschreven in hoodfstuk vijf, behandelt **hoofdstuk negen** de effecten van verschillende ontstekings mediatoren en ß-adrenerge farmaca op de reactiviteit van het adenylyl cyclase in AM van controle-, CARA- en astma patiënten. De basale cAMP gehaltes in AM van CARA patiënten bleek significant hoger te zijn dan van AM van controle patiënten (vgl. antigen challenged/gesensibiliseerd en controle cavia AM). Daarnaast bleek de reactiviteit van het adenylyl cyclase in AM van CARA- en astma patiënten voor deze stimuli verlaagd te zijn. Heterologe desensibilisatie als oorzaak voor deze verlaagde respons wordt in dit hoofdstuk bediscussieerd.
In **hoofdstuk tien** wordt een meer klinische benadering ter hand genomen om de functie van AM in pulmonale ontstekingsprocessen te onderzoeken. De in dit hoofdstuk beschreven studie behandelt de effecten van twee belangrijke anti-astmatische farmaca op het arachidonzuur metabolisme in AM van controle- en CARA patiënten. Salbutamol (een ß-sympaticomimeticum) en theofylline-achtige farmaca worden bij astma en CARA toegepast als bronchusverwijders voor hun direct cAMP-verhogend effect op gladde spieren (relaxatie). In AM stimuleren deze farmaca ook het cAMP dat in deze cellen echter resulteert in een direct effect op de activiteit van het cyclooxygenase (remming) en een indirect effect op het lipoxygenase (stimulering). Stimulatie van het lipoxygenase leidt tot productie van LTB_4, een uiterst chemotactische stof voor secundaire effector cellen. In dit hoofdstuk wordt verder ingegegaan op de vraag in hoeverre dit schadelijk effect van cAMP-verhogende anti-astmatische farmaca een bijdrage levert aan de mogelijke bijwerkingen die deze farmaca veroorzaken bij de behandeling van astma- en CARA patiënten. Daarnaast wordt in deze studie aangetoond dat veranderingen in de concentratie van cyclisch GMP (een andere tweede boodschapper) geen invloed heeft op het arachidonzuur metabolisme van AM.
Ten slotte beschrijft **hoofdstuk elf** een studie waarin de correlatie werd onderzocht tussen het rookgedrag van controle- en CARA patiënten, basale cAMP- en cGMP gehaltes en het spontane en zymosan-gestimuleerde arachidonzuur metabolisme in AM. Hieruit bleek dat veranderingen in het cAMP gehalte (niet het cGMP) geassocieerd zijn met de productie van PGE_2 en LTB_4 en dat deze samenhang negatief beïnvloed kan worden door het rookgedrag. De door zymosan-geïnduceerde

productie van arachidonzuurmetabolieten verloopt via een mechanisme dat zich onderscheidt van het adenylyl cyclase en het guanylyl cyclase.

AC	adenylyl (adenylate) cyclase
ADP	adenosine diphosphate
AM(s)	alveolar macrophage(s)
ANP(F)	atrial natriuretic peptide (factor)
ant.chall.(ac)	antigen challenged
ATE(ate)	atenolol
ATP	adenosine triphosphate
A23187	calcium ionophore
BAL	broncho alveolar lavage
bhr	bronchial hyperresponsiveness
B_{max}	receptor density
cAMP	cyclic 3',5' adenosine monophosphate
CAR	bronchial carcinoma
cGMP	cyclic 3',5' guanosine monophosphate
CIM(cim)	cimitidine
COPD	chronic obstructive pulmonary disease
CSF	colony stimulating factor
CT	cholera toxin
CTRL(ctrl)	control
DAG	1,2-diacylglycerol
DMP	muramyl dipeptide
DNA	desoxyribonucleic acid
DSCG	disodium cromoglycate
ECF	eosinophil chemotactic factor
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme linked immuno sorbent assay
EPO	eosinophil peroxidase
FEV_1	forced expiratoty volume in one second
FGF	fibroblast growth factor
Fig	figure
fMLP	formyl methionyl isoleucyl phenylalanine
FVC	forced vital capacity

GALT	gut associated lymphoid tissue
GBSS	gey balanced salt solution
GDP	guanosine diphosphate
GM-CSF	granulocyte macrophage colony stimulating factor
GMP-PCP	guanylyl (βγ-methylene)-diphosphonate
Gpp(NH)P	guanosine-5'-(β,γ-imino)triphosphate
GTP	guanosine triphosphate
HETE	hydroxy eicosatetraenoic acid
$H_{1(2)}$	type 1(2) histamine receptor
HIST(hist)	histamine
HLA	human leukocyte antigen
HPETE	hydroxy peroxy eicosatetraenoic acid
IBMX	3-isobutyl-1-methyl xanthine
ICYP	iodocyanopindolol
IFN	interferon
Ig	immuno globulin
IGF	insulin-like growth factor
IL	interleukin
INDO(indo)	indomethacin
IP_3	inositol 1,4,5-triphosphate
ISO(iso)	isoprenaline
K_d	dissociation constant
LAK	lymphokine-activated killer
LPS	lipopolysaccharide
Lt	leukotriene
MBP	major basic protein
MEP(mep)	mepyramine
MF	membrane fraction
MIP	macrophage inflammatory protein
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NANC	non-adrenergic non-cholinergic
NCA	neutrophil chemotactic activity
NCF-a	neutrophil chemotactic factor of anaphylaxis

NEP	neutral metalloendopeptidases
NK	neurokinin
ns	not significant
PAF	platelet activating factor
PDE	phosphodiesterase
PDGF	platelet derived growth factor
Pg	prostaglandin
pH	negative logarithm of hydrogen ion concentration
PI	phosphatidyl inositol (phosphoinositide)
PLA_2	phospholipase A_2
PLC	phospholipase C
PMA	phorbol myristate acetate
PMN	polymorphnuclear cells
PROP(prop)	propranolol
RIA	radio immuno assay
SALB(salb)	salbutamol
SEM	standard error of the mean
sens	sensitized
SN	supernatant
SNP	sodium nitroprusside
SOD	superoxide dismutase
SP	substance p
TGF	transforming growth factor
TNF	tumor necrosis factor
Tx	thromboxane
VIP	vasoactive intestinal peptide

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curriculum vitae

Fred Beusenberg was born on september 22nd, 1962, in Hoensbroek. After finishing secundary school (Atheneum B) in 1981, he started his biological study at the University of Amsterdam during which he was involved in three research projects at the Academic Medical Centre (AMC) in Amsterdam: dermatology (Prof. Dr. R.H. Cormane and Dr. W. Westerhof), immunology (Prof. Dr. H.M. Jansen and Dr. T.A. Out) and pharmacology (Dr. R.S. Leeuwin). He attained his masters degree as a medical biologist in 1987. From 1987 till 1991 he was working as a research scientist supported with grants from the Netherlands Asthma Foundation at the Department of Pharmacology (headed by Prof. Dr. I.L. Bonta and Prof. Dr. P.R. Saxena) of the Erasmus University of Rotterdam. In september 1991, he joint Organon International B.V. as a scientific editor at the Department of Regulatory Affairs.
He is married to H.J.I.M. Rodolf, a school teacher and together they have two children, Robin and Nousjka.

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