Heterologous amplification of homologous beta-adrenoceptor desensitization in airway smooth muscle
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Chapter 1

General introduction
Chapter 1

Asthma

Definition of Asthma

Asthma is a chronic inflammatory airway disorder in which many cells and cellular elements play a role, and it is becoming a serious global health problem. People of all ages in countries throughout the world are affected by this disease that can be severe and sometimes fatal [1]. The prevalence of asthma is increasing everywhere, especially among children. Airway inflammation in asthma is associated with airway hyperresponsiveness, airflow limitation and respiratory symptoms. Airflow limitation is the result of acute bronchoconstriction, swelling of the airway wall, chronic mucus formation and airway wall remodelling. For developing asthma, the production of abnormal amounts of IgE antibodies, in response to common environmental allergens, is the strongest identifiable predisposing factor [2]. Bronchial asthma is recognized by recurrent episodes of airflow limitation that are usually reversible either spontaneously or with treatment [3] and is accompanied by symptoms of breathlessness, wheezing, chest tightness and cough, particularly at night or in the early morning. Some patients with asthma show enhanced production of sputum as well. Exacerbations of asthma, including severe attacks or worsening of asthma symptoms, can develop rapidly or gradually. Exacerbations can be severe and may result in death in the absence of proper treatment [2].

Pathophysiology in asthma

Post mortem studies of patients who have died of asthma have revealed that the lung is overinflated. Microscopically there is usually an extensive infiltration of the airway lumen and wall with eosinophils and lymphocytes which is accompanied by vasodilatation, microvascular leakage and epithelial disruption. In addition, trophic changes have also been identified, including smooth muscle hypertrophy, new vessel formation, increased numbers of epithelial goblet cells and the deposition of interstitial collagens beneath the epithelium (basement membrane thickening) [2,4]. It has been demonstrated that both acute and chronic inflammation is irregularly distributed throughout the airways, including the smallest airways and the parenchyma [5]. Studies in living patients with mild asthma, using endobronchial biopsies, generally reflect those seen in autopsy. However, in patients with more severe asthma, it has been demonstrated that not only eosinophils and lymphocytes but also neutrophils are present and may contribute to a more severe disease [6].

Mast cells and eosinophils have been implicated as the key effector cells of the inflammatory response. These cells have the ability to secrete a wide range of mediators that act on the airways both directly and indirectly through neuronal mechanisms [7]. In addition, using immunological and molecular biological techniques, it has been found that T lymphocytes play a pivotal role in orchestrating the inflammatory response by releasing
multifunctional cytokines [8]. Cytokines that are generated by cells of the airways, including fibroblasts and endothelial and epithelial cells, are considered to play a role in the maintenance of the inflammatory response [9]. Cytokines as well as chemokines and growth factors, seem to be very important in mediating the trophic changes of the airways, including hyperplasia of airway smooth muscle, increase in goblet cell number, enlargement of submucous glands and remodeling of the airway connective tissue. These factors are produced by a wide variety of cells, including mast cells, lymphocytes, eosinophils, basophils, epithelial cells, dendritic cells and smooth muscle cells [2].

**Mechanisms of asthma**

*Airway obstruction*

As indicated above, multiple factors are responsible for the airway narrowing in patients with asthma. The major cause of airway narrowing is contraction of bronchial smooth muscle induced by agonists released from inflammatory cells, including histamine, tryptase, prostaglandin D2 and leukotriene C4 from mast cells, neuropeptides from local efferent nerves and acetylcholine from postganglionic parasympathetic nerves [2]. The effect of airway smooth muscle contraction is exaggerated by thickening of the airway wall due to acute edema, infiltration of various cells, chronic hyperplasia of smooth muscle, vascular and secretory cells (remodelling) and deposition of matrix proteins in the airway wall [10]. In addition, even more airflow limitation is observed when the lumen of the airways becomes filled with viscous secretions produced by goblet cells and submucosal glands, leakage of plasma proteins from the bronchial microvasculature and cellular debris [10,11]. As a consequence of airway narrowing airway resistance is increased and maximal expiratory flow is reduced.

*Airway inflammation*

Airway inflammation in asthma is very complex and mechanisms involve a cascade of events mediated by many different kinds of cells, factors and mediators that closely interact with each other [12]. Repeated exposure to allergens may lead to the development of airway inflammation and hyperresponsiveness, in which an IgE-mediated response is considered to play an important role [13-15]. In susceptible individuals the asthmatic reaction after allergen-inhalation can be divided into an early asthmatic reaction (EAR) and a late asthmatic reaction (LAR). The EAR develops rapidly, within minutes after inhalation of the allergen, and is reversible within 1 to 2 h. It is characterised by mast cell degranulation, mucus production and bronchoconstriction [16-18] and is followed by a late phase reaction (6 to 9 h after allergen challenge), which may last for up to 24 h and is characterised by more severe bronchoconstriction, mucus secretion, thickening of the bronchial wall, epithelial shedding and extensive infiltration of CD 4+ T cells and eosinophils in the lung tissue [19-21]. Both mast cells and dendritic cells are thought to be very important in the acute airway response to allergens, whereas infiltration of eosinophils
plays a role both in the early and the late asthmatic reaction [22]. Dendritic cells seem to be key cells for allergen presentation in asthma [23]. These allergens cause cross-linking of the high-affinity IgE receptor FcεR1 and as a consequence the mast cells are activated and degranulate. Inflammatory mediators like histamine, prostaglandins, leukotrienes, platelet-activating factor as well as reactive oxygen species are rapidly released and cause acute bronchoconstriction, mucus hypersecretion, vasodilatation and microvascular leakage [17,24]. Mast cells are therefore very important in the acute airway responses to allergens and may also contribute to remodelling in chronic asthma [25]. During the late asthmatic reaction activated airway cells release cytokines and chemokines into the circulation, stimulating the release of inflammatory leukocytes, especially eosinophils and their precursors, from the bone marrow into the circulation[26]. Activated eosinophils secrete a variety of mediators, including major basic protein (MBP), eosinophilic cationic protein (ECP), cytokines, leukotrienes, PGE2 and PAF, causing bronchoconstriction, epithelial damage and airway hyperresponsiveness [22,27-31].

An important step in the generation of an immune response is the activation of T lymphocytes by antigen presenting cells. T cells can be divided in cytotoxic T cells (CD8+) and helper T cells (CD4+), based on the function of the cells. The latter is involved in the regulation of the effector functions of immune cells by the secretion of cytokines. In the immune system both antibody-mediated and cell-mediated processes can be identified. Antibody-mediated processes involve the production and secretion of specific antibodies by B lymphocytes, while cell-mediated processes depend on T lymphocytes. Two distinct CD4+ T-helper (Th) subtypes (Th1 and Th2) have been characterized on the basis of their profile of cytokine production and it is widely acknowledged that an imbalance between these T-helper cells towards a Th2 activation type may contribute to the development of airway inflammation in asthma [32]. Both subtypes secrete IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), but the Th1 cells preferentially produce IL-2, which stimulates T lymphocyte proliferation, interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα). As mentioned, the Th2 cells are primary involved in asthma and secrete the cytokines IL-4, IL-5, IL-9, IL-13 and IL-16 [33-35]. These cytokines are responsible for the development of the classical delayed-type or cell-mediated hypersensitivity reaction and play a role in IgE production, mast cell activation and eosinophilia. In bronchoalveolar lavage fluid and bronchial biopsies of patients with asthma the expression levels of the cytokines IL-4, IL-5 and IL-13 have shown to be increased compared to healthy controls [8,36-39]. Both IL-4 and IL-13 promote the production of IgE by B cells [40,41] and IL-4 has been implicated in the differentiation of naive T-helper cells (Th0) towards the Th2 subtype [42]. IL-4 has also shown to be a growth factor for mast cells [43] and is involved in mucus production [44]. IL-13 is known to be involved in an enhanced mucus production, airway hyperresponsiveness and eosinophilia [45] and IL-5 has been implicated in development of airway inflammation and hyperresponsiveness by the activation and attraction of eosinophils [46,47].
General introduction

Airway remodeling

Acute inflammatory diseases obviously need various repair processes to restore normal structure and function. In chronic asthma, these processes are disturbed and ineffective repair results in remodelling of several structures. Epithelial damage leads to a loss of its protective barrier, exposing the deeper airway structures to environmental insults. Both inflammatory and structural cells produce growth factors that lead to angiogenesis, proliferation of airway smooth muscle cells, thickening of basement membranes and fibrosis [48]. Increased smooth muscle mass in airways leads to an increase in bronchial responsiveness as a result of increasing force in response to bronchoconstrictor stimuli and reduction of the airway’s diameter [49]. Prolonged epithelial repair, overproduction of profibrotic growth factors, such as TGF-β, and proliferation and differentiation of fibroblasts into myofibroblasts is thought to play an important role in the remodelling process. In addition, activated myofibroblasts produce a wide range of growth factors, chemokines and cytokines. These mediators promote proliferation of airway smooth muscle cells and increases in microvascular permeability [2]. Furthermore, in patients who have died from asthma increased deposition of matrix molecules have been observed deeper in the airway wall.

Under normal circumstances the extracellular matrix is a dynamic structure, which is characterised by an equilibrium between the synthesis and degradation of extracellular matrix components. Matrix metalloproteases are able to selectively degrade extracellular matrix components and play an important role the dynamic equilibrium mentioned above. However, they have also been implicated to play a crucial role in the trafficking of inflammatory and structural cells [2]. Synthesis of different proteins of the extracellular matrix is induced by cytokines and growth factors [50]. Overall, the airways in asthma display various structural alterations that can all contribute to an overall increase in airway wall thickness and airway hyperresponsiveness.

Airway hyperresponsiveness

Airway hyperresponsiveness is a characteristic feature of asthma and involves an increased sensitivity of the airways to inhaled constrictor agonists. In addition, an increased slope of the dose-response curve and a greater maximal response to the agonist is also observed [51]. The severity of airway hyperresponsiveness correlates with the severity of asthma and with the amount of treatment needed to control symptoms [52]. The observed enhanced contractility of airway smooth muscle in patients with asthma could result from alterations in the contractile apparatus [53], in smooth muscle tissue elasticity, or in the extracellular matrix, and seems to be associated with increased velocity of shortening [54]. Furthermore, the mechanisms responsible for exaggerated reactivity may also involve smooth muscle growth [55] and changes in the contractility or phenotype of airway smooth muscle cells, with cells varying between contractile, secretory, and proliferative phenotypes in interaction with airway inflammation [56,57]. Moreover, inflammatory changes in the airway wall could strongly enhance airway narrowing during smooth muscle contraction.
Changes in the organization of contractile filaments or in the plasticity of smooth muscle cells can underlie the maintenance of chronic airway hyperresponsiveness [59,60].

**Figure 1** Several inflammatory cells are recruited and/or activated in the airways, releasing a variety of inflammatory mediators that have acute effects on the airway (such as bronchoconstriction, plasma leakage, vasodilatation, mucus secretion, sensory nerve activation and cholinergic reflex-induced bronchoconstriction), together with structural changes (remodelling) that include subepithelial fibrosis, increased numbers of blood vessels and mucus-secreting cells, and increased thickness of airway smooth muscle as a result of hyperplasia and hypertrophy. Figure adapted from Barnes et al. [61].

**Asthma therapy**

**Overview of therapeutic drugs**

Most asthma guidelines prefer a stepwise approach to asthma treatment. For patients displaying mild asthma with occasional symptoms only, the use of an inhaled β-agonist as needed is preferred. With increasing asthma severity further drugs are added to the treatment (from inhaled corticosteroids and long acting β-agonists to oral corticosteroids eventually) [62]. Corticosteroids are widely used to treat various inflammatory and immune diseases. As discussed above, patients with asthma have a specific pattern of inflammation in the
airways that is characterized by degranulated mast cells, an infiltration of eosinophils, and an increased number of activated Th2 cells. It is believed that this specific pattern of inflammation underlies the clinical features of asthma, including intermittent wheezing, dyspnoea, cough, and chest tightness. Suppression of this inflammation by corticosteroids controls and prevents these symptoms in most patients [63]. Inhaled corticosteroids are especially helpful in patients with mild to moderate asthma and are recommended to patients who need more than one dose of β-agonist inhalation per day [62]. These drugs effectively improve lung function and reduce symptoms and exacerbations in asthmatic patients [64-66]. There is also evidence that early use of inhaled corticosteroids may prevent irreversible airway obstruction and that regular treatment markedly reduces asthma mortality [67]. Inhaled corticosteroids have therefore become very helpful in the treatment of asthma. No evidence has been found that the low doses required by most patients cause clinical important side effects [68]. In contrast, oral corticosteroids cause more morbidity and have many more adverse effects, and with patients on long-term oral steroids the question of prophylaxis against osteoporosis needs to be considered [62].

Theophylline has been used to treat asthma for many years as an add-on therapy for corticosteroids, but its mechanism of action has been difficult to elucidate. Originally, theophylline was used as a bronchodilator, because it relaxes airway smooth muscle by inhibiting phosphodiesterases [69]. In addition, low doses of theophylline have been demonstrated to exert anti-inflammatory effects [70], probably not mediated by phosphodiesterase inhibition because the inhibition of these enzymes is trivial at low plasma concentrations that are clinically effective [71]. Therapeutic concentrations of theophylline markedly potentiate the anti-inflammatory effects of corticosteroids in vitro [72]. This effect may explain why adding a low dose of theophylline is more effective than increasing the dose of inhaled corticosteroids in patients whose asthma is not adequately controlled [73-75]. However, theophylline seems to be less effective than long acting β-agonists as an add-on therapy for corticosteroids [76].

Other possible therapeutic drugs are inhaled anticholinergic agents, such as ipratropium bromide. These agents are bronchodilators that block the effect of acetylcholine released from cholinergic nerves in the airways. In asthma, inhaled anticholinergics are less potent bronchodilators than inhaled β-agonists, and in general, have a slower onset of action. However, they may serve as an alternative bronchodilator for patients who experience adverse effects, such as tachycardia, arrhythmia and tremor, from rapid-acting β-agonists [77].

A new class of asthma medication are cysteinyl leukotriene receptor antagonists. In addition to their potent bronchoconstrictor properties, cysteinyl leukotrienes induce pathophysiologic responses similar to those associated with asthma, including edema and migration of eosinophils [78]. Cysteinyl leukotrienes in asthma (LTC₄, LTD₄ and LTE₄) are likely to be derived predominantly from mast cells and eosinophils, with the greatest production being from surface mast cells in the airways, and the production has been shown to be increased in asthma [79]. Cysteinyl leukotriene receptor antagonists, such as montelukast and zafirlukast, prevent the action of cysteinyl leukotrienes at the type 1
leukotriene receptor. Although these antagonists have had some clinical success in asthma, they are considerably less effective than inhaled corticosteroids [79]. Despite a large number of available medications, the asthma epidemic is continuing to increase. Existing therapies, using β₂-agonists and corticosteroids, provide relief for patients with mild-to-moderate asthma, reversing the bronchoconstriction and decreasing the inflammation, but these therapies provide little relief for chronic asthmatics. In particular, new therapy is needed for severe asthma that is poorly controlled by high doses of corticosteroids, as well as agents to counter acute emergency asthma. There are numerous therapies in clinical development that combat the inflammation found in asthma, specifically targeting eosinophils, IgE, adhesion molecules, cytokines (interleukin-4, -5, -13) and chemokines, inflammatory mediators, and cell signalling (kinase inhibitors). However, much is still to be learned about the mechanisms involved in the development and treatment of chronic asthma [61,80,81].

\textit{β₂-adrenoceptor agonists}

Inhaled β₂-adrenoceptor agonists are by far the most effective bronchodilators used for the relief of symptoms in patients with asthma [82]. β₂-Agonists are able to relax airway smooth muscle, enhance mucociliary clearance, decrease vascular permeability and may modulate mediator release from mast cells and basophils [83]. Short acting β₂-agonists, such as salbutamol and terbutaline, are very effective in relieving acute attacks of asthma. However, they do not provide benefit when given regularly [84] and it has been described that patients can even deteriorate [85]. As a consequence, short acting β₂-agonists should only be used as required. Moreover, excessive use of these β₂-agonists may result in increased asthma deaths [86]. In contrast, regular use of long acting β₂-agonists, such as salmeterol and formoterol, improves asthma control, reduces asthma exacerbations and provide long term protection against bronchoconstrictor stimuli [64,65,87]. Long acting β₂-agonists are more and more introduced at an earlier point in asthma management and can be given in combination with an inhaled corticosteroid. Fixed combination inhalers of long-acting β₂-agonists and corticosteroids are available and seem to be the most effective way to control asthma because these two classes of drugs have complementary and synergistic effects [88]. The expression of β₂-adrenergic receptors in the lung is increased by corticosteroids and downregulation and uncoupling in response to β₂-agonist stimulation is inhibited [89-91]. In addition it has been demonstrated that β₂-agonists enhance the action of corticosteroids by increasing the translocation of glucocorticoid receptors [92] and enhancing the suppression of inflammatory genes [93,94].
Pharmacology of bronchoconstriction

Airway smooth muscle

The traditional view of airway smooth muscle in asthma was of a passive partner in airway inflammation, contraction and relaxation, but nowadays airway smooth muscle has been found to have a number of properties and functions which contribute to asthma pathogenesis. It undergoes changes in contractile and relaxant properties in response to asthmatic mediators and cytokines and airway smooth muscle remodeling is induced by growth factors which are present in the asthmatic airways. Airway smooth muscle cells can also have synthetic properties and are a rich source of cytokines and chemokines. In addition, airway smooth muscle cells can produce inflammatory mediators which act to alter contractility and the proliferation response in an autocrine manner. The production of chemokines by airway smooth muscle cells may be involved in an exaggerated inflammatory response [95] and evidence suggests that changes in airway smooth muscle phenotype may play a fundamental role in the pathogenesis of lung diseases such as asthma [96]. However, despite all these properties of airway smooth muscle cells, it is most likely that changes in airway smooth muscle tone are by far the most important factor in determining airflow [97].

Receptors

Most bronchoconstrictors activate G protein-coupled receptors on the cell membrane of airway smooth muscle cells that are linked to the contractile-apparatus through different signal transduction pathways. In asthma, many bronchoconstrictor mediators are produced by inflammatory cells or released from airway nerves. Agonists, such as acetylcholine, histamine, cysteinyl leukotrienes and thromboxane, that activate receptors on airway smooth muscle cells are direct bronchoconstrictors. However, bronchoconstriction can also be induced indirectly by agonists that release constrictor agents from other cells. For example, adenosine and allergens can cause bronchoconstriction by releasing mediators, such as histamine and cysteinyl leukotrienes, from mast cells, and bradykinin may indirectly constrict airway smooth muscle by releasing bronchoconstrictors, such as acetylcholine and tachykinins, from airway nerves [97]. Histamine-induced bronchoconstriction is mediated via H₁ receptors [98], whereas thromboxane and other bronchoconstrictor prostaglandins, such as PGD₂ and PGF₂α, are acting via a TP receptor in human airways [99,100]. Another important group of constrictors in human airways are cysteinyl leukotrienes. Leukotriene C₄, D₄ and E₄ all act through a common cysteinyl leukotriene I receptor [101]. Many neuropeptides, which are found in the nerves of human airways, are able to constrict airway smooth muscle. For example, tachykinins cause bronchoconstriction through the activation of NK₂-receptors [102]. Bradykinin-induced
bronchoconstriction is mediated through bradykinin B2-receptors on airway smooth muscle cells [103]. The parasympathetic nervous system is the major bronchoconstrictor neural pathway in the airways. Acetylcholine, the principal neurotransmitter of the parasympathetic nervous system, leads to airway smooth muscle contraction upon muscarinic receptor stimulation, and is one of the most efficacious contractile agents of airway smooth muscle. Four different subtypes of muscarinic receptors (M1-M4) have been identified in airway tissues [104]. However, no evidence is found for the M4-receptor subtype in human lung [105]. M1 receptors are mainly located at parasympathetic ganglia, where they facilitate neurotransmission. M2 receptors are present in both postganglionic nerves, to control neurotransmitter release, and smooth muscle cells, and M3 receptors are primarily located at the airway smooth muscle, glands and epithelial cells [106,107]. The receptor subtype mediating bronchoconstriction is the M3-receptor [108], which is coupled to a stimulatory G-protein (Gq). M2-receptors are coupled via an inhibitory G-protein (Gi) to inhibit adenylyl cyclase, which would predict to have a bronchoconstrictor effect. However, although the M2-receptor predominates over the M3-receptor (in an approximate 80% to 20% ratio) in airway smooth muscle, it has been difficult to demonstrate any functional role of M2 receptors [109-111].

**Signal transduction**

*Phosphoinositide metabolism*

Most of the bronchoconstrictor receptors discussed above, including H1 and M3-receptors, are coupled via the heterotrimeric G-protein Gq. Upon agonist binding, the receptor undergoes a conformational change that promotes its association with Gq in its GDP-bound inactive state. The receptor especially interacts with the α subunit of the G-protein heterotrimer, resulting in the release of GDP and subsequent binding of GTP. This exchange of GDP for GTP induces a conformational change of Gα and causes the dissociation of the active GTP-bound Gα from the Gβγ dimer and in turn activates an effector molecule [112]. The membrane-associated enzyme phospholipase C (PLC) is the principle effector of Gq-mediated signalling. Eleven different isoforms of PLC have been identified, but members of the PLCβ subfamily tend to mediate the actions of activated Gq [113]. PLCβ promotes the hydrolysis of phosphoinositide (4,5)biphosphate (PIP2) into inositol (1,4,5)trisphosphate (IP3) and 1,2 sn-diacylglycerol (DAG) [114-117]. Subsequently, IP3 diffuses into the cytosol and binds to a specific IP3-receptor (IP3,R) on the endoplasmic/sarcoplasmic reticulum, which leads to the release of Ca2+ from intracellular stores. The rapid and transient increase in intracellular Ca2+ is followed by a sustained influx of extracellular calcium [118]. The increase in intracellular calcium is thought to activate the contractile apparatus and to induce airway smooth muscle contraction. Indeed, in animal [103,119-121] and human [122] airway smooth muscle it has been demonstrated that the phosphoinositide metabolism induced by various
bronchoconstrictors, including muscarinic agonists, is involved in the pharmacomechanical coupling of contraction. In bovine tracheal smooth muscle a direct relationship was found between the efficacy of different muscarinic agonists to induce inositol phosphates accumulation and their potency to induce contraction. A remarkable reserve of inositol phosphates production was found for full agonists (methacholine, oxotremorine), and no reserve for the partial agonist McN-A-343 [120]. As mentioned above, the formation of IP$_3$ parallels the production of DAG [123], which remains close to the plasmamembrane and has been shown to activate protein kinase C (PKC) by causing it to translocate to the cell membrane and by increasing its sensitivity to Ca$^{2+}$ [124]. Activated PKC is then capable of phosphorylating various cell membrane-associated proteins, including some receptors, G-proteins and regulatory proteins and has been implicated in the tonic phase of contraction [125]. In addition, it has been demonstrated that activation of PKC exerts a feedforward control of both the methacholine- and histamine-induced Ca$^{2+}$-mobilization and influx, which suggests that PKC may be involved in the phasic contraction as well [126].

**Pharmacomechanical excitation-contraction coupling**

The protein components of the contractile apparatus of airway smooth muscle are similar to those found in other smooth muscles and include actin, myosin, calmodulin, tropomyosin, caldesmon and calponin. In response to constrictor stimuli in smooth muscle, the intracellular Ca$^{2+}$ concentration increases and the activator Ca$^{2+}$ binds to the acidic protein calmodulin. Subsequently this complex activates myosin light chain kinase (MLCK), which rapidly phosphorylates the 20-kDa light chains of myosin (MLC 20), enabling the molecular interaction of myosin with actin. If the intracellular Ca$^{2+}$-concentration remains above basal level, myosin phosphorylation remains elevated as well. Energy released from ATP by myosin ATPase activity results in the cycling of the myosin cross-bridges with actin for contraction [127-130]. Ca$^{2+}$ probably also activates multifunctional Ca$^{2+}$/calmodulin-dependent kinase, which might phosphorylate other putative regulatory proteins such as caldesmon or calponin. Rapid phosphorylation of these proteins might relieve inhibition of myosin ATPase. Tropomyosin is an actin filament regulatory protein, which appears to modulate phosphorylation of myosin ATPase along with myosin light chain phosphorylation, possibly in concert with caldesmon and calponin [131]. In addition to the Ca$^{2+}$-dependent activation of MLCK, the state of myosin light chain phosphorylation is further regulated by myosin light chain phosphatase (MLCP), which removes the high-energy phosphate from the light chain of myosin to promote smooth muscle relaxation [132].

**PKC**

PKC is a multifunctional protein kinase that phosphorylates serine and threonine residues in many target proteins. Rather than being a single protein, PKC is known to comprise a large family of enzymes that differ in structure, cofactor requirements and function. PKC is a superfamily including three types of isoforms. The conventional isoforms ($\alpha$, $\beta_1$, $\beta_2$, $\gamma$)
require Ca\textsuperscript{2+} and DAG to become activated, whereas the novel isoforms (\(\delta\), \(\iota\), \(\varepsilon\), \(\theta\), \(\mu\)) are Ca\textsuperscript{2+}-insensitive and only require DAG. The atypical isoforms (\(\zeta\), \(\tau\), \(\lambda\)) are calcium- and DAG-insensitive and are activated by phosphatidylserine [133,134]. As all isoforms of PKC posses a binding site for phosphatidylserine, conventional and novel PKC isoforms can also be activated by phosphatidylserine. The general structure of a PKC molecule consists of a catalytic and a regulatory domain found at the C- and N-terminus respectively. Activation of PKC involves translocation from the cytosol to the cell membrane [124]. This membrane association is Ca\textsuperscript{2+}-dependent and specific anchoring proteins, including “receptors for activated C-kinase” (RACKS) localize the kinases at their sites of action [134]. With respect to conventional and novel PKC’s, the availability of DAG is essential for their activation, as it facilitates the penetration of these PKC’s into the cell membrane [135]. Activation of conventional PKC’s is critically dependent on the intracellular calcium concentration and needs a transient increase in calcium to promote translocation of the inactive PKC to the cell membrane. Resting Ca\textsuperscript{2+}-levels are insufficient to activate PKC [136].

The expression and distribution of PKC isoforms varies markedly between cells and tissues [137]. PKC \(\alpha\), \(\beta\), \(\beta_2\), \(\delta\), \(\varepsilon\) and \(\zeta\), but not \(\gamma\) or \(\iota\) are expressed in bovine tracheal smooth muscle [138], whereas PKC \(\alpha\), \(\beta\), \(\beta_2\), \(\delta\), \(\varepsilon\), \(\theta\), \(\iota\), \(\zeta\), \(\tau\) and \(\mu\) have each been identified in human tracheal smooth muscle [138,139]. Conventional and novel PKC’s can be activated directly by phorbol esters, such as phorbol myristate acetate (PMA), which have therefore been useful in examining the role of PKC. Unfortunately, theoretically phorbol esters have the ability to activate all conventional and novel PKC isoforms present in airway smooth muscle, whereas receptor agonists, almost certainly, will selectively activate specific isoforms, including atypical PKC’s. Another possibility to study the role of PKC is the use of PKC-inhibitors. Frequently used inhibitors are staurosporin and Ro31-8220, which are not very selective for PKC. GF 109203X, however, is a very selective inhibitor of novel and conventional PKC isoforms and Gö6976 specifically inhibits conventional PKC isoforms [140,141]. Unfortunately, the differential function of PKC isoforms is not clearly understood, because isoform-selective inhibitors are not yet available.

PKC has been proposed to be a potential mediator of tonic contraction of airway smooth muscle. Thus, in airway smooth muscle from the cow [125,142,143], guinea-pig [144], rabbit [145], rat [146] and human [147,148], low concentrations of phorbol esters elicited a slow, tonic contraction, and exposure of airway smooth muscle strips to 4\(\beta\)-PDBu and carbachol resulted in the phosphorylation of five contractile proteins (caldesmon, filamin, synemin and \(\alpha\)- and \(\beta\)-desmin), which completely paralleled the development of force [125,142]. In addition, using bisindolylmaleimide derivatives as inhibitors of PKC, including staurosporine, Ro-318220, Ro-31-7549 and GF 109203X, it has been demonstrated that the contraction induced by phorbol esters and some Ca\textsuperscript{2+}-mobilizing agents was antagonized [146,149,150]. Additional data from the studies mentioned above indicated that phorbol ester-induced contraction of airway smooth muscle by PKC-dependent processes involves an increase in the open state probability of L-type voltage dependent calcium channels, Ca\textsuperscript{2+}-influx, MLC 20 phosphorylation and activation of the
contractile machinery. Moreover, it has been demonstrated that phorbol esters can contract smooth muscle by inhibiting the activity of MLCP, leading to MLC 20 phosphorylation and force development at a constant Ca²⁺-concentration [151-153]. This phenomenon is known as Ca²⁺-sensitization [154]. Studies carried out in rabbit tracheal smooth muscle have demonstrated that phorbol esters not only induced contraction, but at high concentrations also produced smooth muscle relaxation [145]. It was proposed that low levels of PKC activation contribute to enhanced Ca²⁺-influx and maintenance of tone, whereas high degrees of activation stimulate the electrogenic Na⁺-K⁺ pump leading to relaxation. Besides its important role in airway smooth muscle contraction, PKC has also been implicated in the cross-talk between the phosphoinositide metabolism and the β-adrenergic receptor (see paragraph ‘regulation of the β₂-adrenoceptor’).

Intracellular calcium homeostasis

Regulation of intracellular calcium

Regulation of the intracellular calcium concentration in smooth muscle cells is a highly complex phenomenon. Under resting conditions only few Ca²⁺-ions enter the cell and the intracellular calcium concentration is maintained relatively constant due to different homeostatic mechanisms. These includes a plasma membrane Na⁺-Ca²⁺ exchanger driven by the Na⁺ gradient (maintained by the Na⁺-K⁺-ATPase), a Mg²⁺-dependent plasma membrane Ca⁺-H⁺-ATPase and a sarcoplasmic reticulum Ca²⁺-ATPase [154]. As discussed before, contractile agonists such as acetylcholine and histamine require an elevation of intracellular calcium to induce airway smooth muscle contraction. This elevation of intracellular calcium involves various second messengers inside the cell and results from several mechanisms, including influx from the extracellular space and release from intracellular stores, principally the sarcoplasmic reticulum (SR) [155]. Upon contractile agonist stimulation a characteristic biphasic profile of intracellular calcium levels is observed in airway smooth muscle [118,156-160]. First, there is an initial rapid rise in intracellular calcium level, which reaches its maximum within 10-15 s and then rapidly declines. This transient rise in intracellular calcium is dependent upon the release of calcium from intracellular stores, and is mediated by agonist-induced IP₃ production and subsequent stimulation of IP₃ receptors in the sarcoplasmic reticulum membrane [114,161,162]. The precise mechanism through which the calcium concentrations subsequently decline is less clear, although reuptake into stores is the most likely explanation. Plasma membrane calcium pumps can extrude calcium from the cell but time course of the observed response seems too rapid for this mechanism to be important [163]. Second, in the continued presence of the agonist, calcium levels do not completely return to baseline levels, but a sustained plateau response is usually observed [118]. This sustained calcium response is important for maintaining the contractile response to the agonist, and it is clear that the source of this calcium is extracellular Ca²⁺-influx [118,164]. Thus, it has been demonstrated in airway smooth muscle that in the absence of extracellular calcium the
plateau phase is lost [118,159,160]. The amplitude of the calcium transient and the level of the subsequent plateau have been shown to depend on contractile agonist concentration [158,159,165].

Several models for extracellular Ca\(^{2+}\)-influx in smooth muscle cells have been proposed. In airway smooth muscle, Ca\(^{2+}\)-influx may occur through voltage-gated (VOCC) [166,167], receptor gated (ROCC) [118] and store-operated calcium channels (SOCC) [168,169]. However, despite their established presence, it has been demonstrated that contractile agonist-induced calcium entry is relatively insensitive to inhibitors of voltage-operated Ca\(^{2+}\) channels [166,170,171] in airway smooth muscle. In most instances, the signal for Ca\(^{2+}\)-entry is somehow derived from the IP\(_3\)-mediated depletion of calcium from intracellular stores, a process called “capacitative calcium entry” or “store-operated calcium entry” [172,173], thus allowing for replenishment of intracellular Ca\(^{2+}\)-stores. Two major categories of mechanisms to activate capacitative calcium entry have been hypothesized. Perhaps the simplest mechanism for linking intracellular stores to the plasma membrane is through the release of a diffusible messenger called calcium-influx-factor [174,175]. However, the more favoured model for capacitative calcium entry is conformational coupling, which is a more direct communication with the endoplasmic membrane channels with closely underlying IP\(_3\) receptors [176,177]. There is now considerable evidence for SOCC in smooth muscle [178-180], including airway smooth muscle [169], and it has also been suggested that Ca\(^{2+}\)-influx mediated via SOCC is involved in actual smooth muscle contraction [181]. Furthermore, inhibitors of sarcoplasmic reticulum ATPase have been shown to increase intracellular Ca\(^{2+}\) in human bronchioles and bovine airway smooth muscle, suggesting the involvement of SOCC [182]. A family of proteins that have been considered as candidates for capacitative calcium entry are transient-receptor-potential channels (TRPC) [181,183,184]. Expression of the TRPC genes have been demonstrated crucial for store-operated Ca\(^{2+}\)-influx in mammalian cells, suggesting that TRPC-encoded proteins are the putative SOCC responsible for the observed Ca\(^{2+}\)-influx [182,185,186]. It has been demonstrated in rat airway smooth muscle cells that SOCC potentially involve TRPC1-type Ca\(^{2+}\)-channels [182]. Recently, in Drosophila S2 cells, a new key player has been discovered in the process of capacitative calcium entry, namely the protein stromal interaction molecule (STIM). STIM1 is located both on the plasma membrane and intracellular membranes, and this protein may be responsible for bridging the two [187].

**Intracellular Ca\(^{2+}\)-oscillations**

With the advent of real-time confocal microscopic imaging techniques, it has been shown for many contractile agonists in airway smooth muscle that the first transient rise in intracellular calcium, is followed either by a steady state rise in intracellular calcium or by successive transient calcium peaks, the so called calcium oscillations [188-191]. The occurrence of an oscillatory or non oscillatory calcium response in airway smooth muscle cells depends on various factors, including species variation. It has been suggested that in a
single species, different cell phenotypes, one characterized by oscillatory responses and the other by non oscillatory responses, may coexist. For example, in rat tracheal smooth muscle, about 50% of the cells exhibit muscarinic agonist-induced intracellular Ca\(^{2+}\)-oscillations [190]. The frequency of Ca\(^{2+}\)-oscillations has been shown to depend on the contractile agonist concentration. Thus, in airway smooth muscle higher concentrations of contractile agonists induced a higher frequency of Ca\(^{2+}\)-oscillations [188,190-195]. In addition, the frequency of Ca\(^{2+}\)-oscillations has been correlated to the amplitude of the mechanical response towards contractile agonists [188,195,196]. Over the years, the origin of and the mechanisms involved in these Ca\(^{2+}\)-oscillations have been studied extensively in airway smooth muscle cells, and it has become acknowledged that ryanodine receptors (RyR), located on the sarcoplasmic reticulum, play a major role [168,191,197-200]. The initiation of Ca\(^{2+}\)-oscillations appears to be critically dependent on the generation of IP\(_3\) and calcium release through IP\(_3\) receptor channels. However, once initiated the intracellular calcium oscillations are sustained even in the presence of an IP\(_3\) receptor antagonist and abolished in the presence of RyR antagonists [189,198], indicating that the sustained intracellular calcium oscillations are primarily caused by activation of RyR. Furthermore, as demonstrated in porcine tracheal smooth muscle, these oscillations originate in a certain localized region of the cell and propagate, indicating that calcium release most likely occurs by stimulating calcium release from adjacent regions [194].

This calcium induced calcium release (CICR) has been shown to involve the activation of RyR channels [196]. The RyR channel is activated by very low calcium concentrations, low pH, millimolar ATP and millimolar concentrations of caffeine. Although an endogenous ligand for RyR channels has remained elusive, it has recently been postulated that cyclic ADP-ribose (cADPR), which is a cyclized derivative of \(\beta\)-nicotinamide adenine dinucleotide (NAD) metabolism, is one of the candidate molecules involved in the activation of RyRs and modulation of CICR [201]. Recent studies have provided evidence that calcium release through RyR channels is sensitized not only to calcium but also to cADPR [202-205]. Furthermore, it has been demonstrated in airway smooth muscle that the frequency of intracellular calcium oscillations can be modulated by cADPR and abolished by the cADPR antagonists 8-Amino-cADP and 8-bromo-cADP. Moreover, these antagonists inhibited the global intracellular calcium responses elicited by acetylcholine, bradykinin, entothelin-1 and thrombin [206-208]. In addition, the cADPR-induced calcium release is also inhibited by the RyR antagonists ryanodine and ruthenium red [203]. Together, these data indicate the involvement of cADPR in the calcium release through RyR channels during agonist activation of airway smooth muscle and suggest that cADPR acts as a second messenger in agonist-induced intracellular calcium elevation. CD38, which is a bifunctional protein possessing both ADP-ribosyl cyclase and cADPR hydrolase activities, appears to be the primary source for cADPR in airway smooth muscle [209], by converting \(\beta\)-NAD to cADPR using its ADP-ribosyl cyclase activity. Thus, in isolated airway smooth muscle cells from CD38-deficient mice, intracellular calcium responses to agonists were attenuated compared with cells isolated form wild type mice [210] and downregulation of CD38 expression by antisense CD38 in human airway smooth muscle
cells resulted in attenuated calcium responses to various agonists [209]. Interestingly, there appears to be a receptor and/or receptor-subtype specificity in the use of cADPR-mediated calcium release [202,211]. This was illustrated by the observation that a cADPR antagonist inhibited acetylcholine-induced and endothelin-1-induced, but not histamine-induced, calcium responses in porcine airway smooth muscle [211]. Furthermore, the CD38/cADPR pathway is associated with activation of M₂, but not M₃, muscarinic receptors [211]. One possibility for this receptor or receptor-subtype specificity is the involvement of specific G-proteins in the activation of CD38/cADPR signaling. However, the mechanism of G-protein association with the CD38/cADPR signaling needs further investigation.

Calcium sensitization

As discussed above, activation of smooth muscle involves the generation of a Ca²⁺-signal with unique temporal and spatial properties finally leading to the phosphorylation of MLC 20 and subsequent contraction. However, it has been shown that intracellular Ca²⁺-levels not always correlate with the level of MLC 20 phosphorylation and contraction. Moreover, besides increasing intracellular calcium concentrations, contractile agonists may also increase the Ca²⁺-sensitivity of the contractile machinery [212]. It is widely accepted that the degree of MLC 20 phosphorylation is the essential factor that determines the extent to which smooth muscle contracts. MLC 20 phosphorylation promotes smooth muscle contraction whereas MLC 20 dephosphorylation, following a reduction in the intracellular calcium concentration, results in muscle relaxation. The state of MLC 20 phosphorylation, however, is, in addition to the Ca²⁺-dependent activation of MLCK, also regulated by MLCP which removes the high-energy phosphate group from the light chain of myosin to promote smooth muscle relaxation. Consequently, the MLCK-to-MLCP-activity ratio is the major determinant of the extent of MLC 20 phosphorylation and subsequent contraction [212]. MLCP consists of three subunits, including a myosin-binding subunit. This myosin-binding subunit, when phosphorylated, inhibits the enzymatic activity of MLCP, allowing the light chain of myosin to remain phosphorylated, and thereby promoting contraction [127].

The small G-protein RhoA and its downstream target Rho kinase have been shown to play an important role in the regulation of MLCP activity [210,212,213]. Thus, in smooth muscle cells it has been demonstrated that agonist-induced activation of the RhoA/Rho kinase pathway enhanced MLC 20 phosphorylation at a fixed level of intracellular calcium. Consequently, despite the fact that the intracellular calcium concentration remained unchanged, these agonists augmented the level of contraction [214]. This increased calcium sensitivity of the contractile machinery towards contractile agonists is referred to as Ca²⁺-sensitization. Rho-kinase is able to promote the phosphorylated state of MLC 20 by a direct phosphorylation of MLC 20 [213], or by phosphorylating the myosin-binding subunit of MLCP, thereby inhibiting its activity [153,215]. However, recent studies have shown that it is the Rho-kinase-mediated inactivation of myosin phosphatase, rather than the direct
phosphorylation of MLC by Rho-kinase, that would be responsible for Ca\(^{2+}\)-sensitization of smooth muscle [153].

A number of studies have shown that Rho-kinase is involved in airway smooth muscle contraction induced by either contractile agonists [153,216-220] or KCl [220,221], and it has even been suggested that Rho-kinase may have a pathophysiology-primed role in asthma. Thus, in repeated allergen-challenged rats an augmented role of Rho-kinase in acetylcholine-induced bronchial smooth muscle contraction has been shown [218]. In addition, it has been demonstrated in guinea pigs that active allergic sensitization by itself, i.e. without subsequent allergen exposure, was sufficient to enhance Rho-kinase-mediated airway smooth muscle contraction both \textit{in vivo} and \textit{ex vivo} [222]. Several compounds such as Y27632 and fasudil have been developed and found to inhibit the activity of Rho-kinase specifically which proved very useful for evaluating Rho-kinase functions in smooth muscle. Currently, it is well established that RhoA, and in turn Rho-kinase, can be activated by a variety of G-protein coupled receptors, particularly by those coupled to G\(_{12/13}\), through an interaction with guanine nucleotide exchange factors (RhoGEF’s) [223]. In addition, it was found recently that agonist-stimulated G\(_{q/11}\)-coupled receptors can also activate RhoA, involving the G\(_{q/11}\)-selective GEF, p63RhoGEF [224]. Another possible mechanism for Rho-kinase activation by contractile agonists coupled to G\(_{q}\)-proteins has been demonstrated by the observation that receptors of the vasoconstrictive agonists angiotensin II, endothelin and vasopressin are coupled to G\(_{12/13}\) as well as to Gq [225].

In addition, PKC, which is activated by G\(_{q}\) coupled receptors, has been shown to cause enhanced contraction of vascular smooth muscle even when a rise in intracellular calcium is absent [226,227]. One mechanism for this Ca\(^{2+}\)-sensitization is related to an increase in the level of MLC 20 phosphorylation, which can occur directly by PKC-mediated phosphorylation or indirectly by PKC-mediated inhibition of MLCP. It has been demonstrated that PKC-induced inhibition of MLCP is mediated by CPI-17. CPI-17 is another potential mediator of Ca\(^{2+}\)-sensitization and its phosphorylation by PKC enhances its potency for inhibiting MLCP [228,229]. PKC-dependent and Rho kinase-dependent Ca\(^{2+}\)-sensitization may not be independent pathways. For example, CPI-17 can be phosphorylated by Rho-kinase [229] and conversely, arachidonic acid, which is generated by phospholipase A\(_{2}\), can, either directly or indirectly as a consequence of PKC activation, activate Rho kinase [230-233]. Therefore, it is possible that the mechanisms downstream of PKC and Rho kinase converge at the vicinity of MLCP. In rabbit aorta smooth muscle it has been recently reported, using the Rho-kinase inhibitor fasodil, that Rho kinase was indeed involved in PKC-dependent Ca\(^{2+}\)-sensitization [234].
Pharmacology of bronchodilatation

Airway smooth muscle relaxation

Several endogenous agents act as bronchodilators, either by activating bronchodilator receptors on airway smooth muscle, or via the release of endogenous bronchodilators such as NO or PGE₂ [97]. The best-studied bronchodilators are β₂-agonists, which can activate β₂-adrenergic receptors on airway smooth muscle (see paragraph 'β₂-adrenoceptor mediated relaxation') [235]. Another bronchodilator, PGE₂, which may act as an important regulator of airway smooth muscle tone, especially in asthma, relaxes airway smooth muscle via some EP receptors of which several subtypes have been characterized. PGE₂ may be released from epithelial cells and airway smooth muscle cells and is able, in higher concentrations, to reduce the bronchoconstriction response seen in asthma [97]. Vasoactive intestinal peptide (VIP) has been demonstrated to be a potent relaxant of human bronchi in vitro, but is an ineffective bronchodilator in vivo as it is rapidly metabolized in the airways [236,237]. NO is a direct relaxant of the airways and is a neurotransmitter of bronchodilator nerves [238]. Under basal conditions constitutive NO synthase (cNOS) isozymes present in the airway epithelium and in nonadrenergic noncholinergic (iNANC) nerves synthesize small amounts of NO. cNOS isozymes present in the epithelium are activated by various contractile agonists, whereas the cNOS isozyme in iNANC nerves is activated by depolarization. Epithelium- and nerve-derived NO induce relaxation of the airway smooth muscle by increasing the production of cGMP and/or opening of Ca²⁺-activated K⁺ channels [239].

The β₂-adrenoceptor

At least three β-adrenoceptors are now recognized and cloned [240-243], namely β₁, β₂ and β₃. These subtypes were originally identified in cardiac, vascular and adipose tissue, respectively. Successful cloning of these receptors made it possible to elucidate the structure of the receptor protein, the way in which the receptor interacts with β-agonists and the signal transduction involved. The β₂-adrenoceptor is a member of the 7-transmembrane family of G protein-coupled receptors and is composed of 413 amino acid residues [235]. The seven clusters of membrane-spanning helices are connected by alternating intra- and extracellular loops, with the amino (N)-terminal exposed to the outside and the carboxy (C)-terminal to the inside of the cell [235]. β₂-Adrenoceptors are widely distributed, occurring not only in airway, vascular and intestinal smooth muscle cells, but also in epithelial and endothelial cells and in inflammatory cells, such as T cells, mast cells, macrophages, eosinophils and neutrophils [235]. A major effect of β₂-adrenoceptor stimulation in the airways is smooth muscle relaxation. However, β₂-adrenoceptor stimulation can also result in an increase in mucociliary clearance, vasodilatation and inhibition of vascular leakage. In vitro experiments have shown that β₂-adrenoceptor
stimulation also has inhibitory effects on human inflammatory cells, influencing cell activation and mediator release, cell adhesion and chemotaxis, and cell survival [235,244,245]. In animal and human airway smooth muscle, autoradiographic studies have confirmed the presence of β-adrenoceptors from the trachea down to terminal bronchioles. In some species both β₁- and β₂-receptor subtypes have been demonstrated functionally in airway smooth muscle, the presence of β₁-adrenoceptors being related to the presence of sympathetic innervation of airway smooth muscle [246-250]. The fact that human airway smooth muscle has no functional sympathetic innervation is consistent with both findings that only β₂-adrenoceptors are expressed in all levels of human airway smooth muscle [250,251] and that mRNA expression is absent for β₁-adrenoceptors in human airway smooth muscle [252]. Moreover, relaxation of human central and peripheral airway smooth muscle has shown to be mediated by a homogenous population of β₂-adrenoceptors [253,254]. Autoradiographic and radioligand binding studies have shown that β₂-adrenoceptor density increases with increasing airway generation, thus being greater for small than for large airways, with high levels in the alveolar region [246,251].

**β₂-adrenoceptor mediated relaxation**

*Signal transduction*

The β₂-adrenoceptor belongs to the group of G protein-coupled receptors which are coupled with the heterotrimeric G-protein Gs, and its signalling and regulation have been extensively studied in numerous cells, including airway smooth muscle [112,235,255-257]. Stimulation of the β₂-adrenoceptor results in Gs dependent activation of the enzyme adenylyl cyclase (AC) in a manner which is similar to the activation of PLC via Gq-coupled receptors. Thus, upon binding of the agonist to the β₂-adrenoceptor, GTP binds to the α subunit of the heterotrimeric Gs-protein complex, resulting in the dissociation of the (GTP-occupied) αs-subunit from the βγ subunit. Subsequently, the αs subunit binds to and activates AC, which catalyses the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), subsequently leading to the activation of the cAMP dependent protein kinase A (PKA) [258,259]. cAMP binds to the regulatory subunit of PKA as a consequence of which these subunits dissociate from and thereby activate the catalytic subunit of PKA. Activated PKA is able to phosphorylate key intracellular proteins and is responsible for the majority of the physiological responses to stimulation of β₂-adrenoceptors [260]. MLCK is one of the proteins that can be phosphorylated by PKA [261], which leads to a decreased affinity of MLCK for Ca²⁺/calmodulin, resulting in a decrease in smooth muscle contraction. PKA also phosphorylates large conductance Ca²⁺-dependent K⁺ channels (Maxi-K channels), increasing their open-state probability (and therefore K⁺-efflux), which results in hyperpolarization and relaxation of airway smooth muscle. β₂-Adrenoceptor stimulation of Maxi-K channel activity may also occur independently of PKA, presumably by a direct interaction of Gs and the Maxi-K channel [257,262-264]. Other mechanisms underlying β₂-adrenoceptor-mediated airway smooth
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muscle relaxation include activation of sacrolemmal Ca\textsuperscript{2+}-ATPase [265], augmentation of Na\textsuperscript{-}Ca\textsuperscript{2+} exchange [266,267] and stimulation of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase [268], all resulting in an enhanced extrusion of Ca\textsuperscript{2+} across the plasma membrane or enhanced uptake of calcium into intracellular stores.

In addition to cell relaxation, $\beta_2$-adrenoceptor-mediated activation of PKA also leads to phosphorylation of the transcription factor cAMP response element (CRE) binding protein (CREB), which has been found to induce changes in the transcription (increase as well as decrease) of many genes, including transcriptional autoregulation of the $\beta_2$-adrenoceptor gene [269,270]. Expression of the $\beta_2$-adrenoceptor gene is under control of CRE in the promoter region of the gene and is recognized by CREB. PKA phosphorylation of CREB permits CREB to bind to CRE, followed by its binding to CREB binding protein (CBP). CBP binding leads to the recruitment of the basal transcriptional apparatus to initiate gene transcription, thereby increasing the transcription of the gene [269,271]. Moreover, activation of CREB may modulate changes in the responses to activation of other transcriptional factors that also interact with CBP, possibly as a result of competition over transcription factors.

In addition to the activation of PKA, evidence from several laboratories indicated that cGMP-dependent protein kinase (PKG) may be activated by physiological increases in cAMP [259,272], leading to relaxation of smooth muscle by decreasing the Ca\textsuperscript{2+}-sensitivity of the contractile machinery [273] and activation of Maxi-K channels [274,275]. In HEK293 cells PKA-mediated phosphorylation of the $\beta_2$-adrenoceptor itself has been shown to decrease the coupling efficiency to $G_s$, thereby reducing cAMP production in response to further stimulation. In addition, PKA-induced phosphorylation also resulted in a switch of the $\beta_2$-adrenoceptor coupling from $G_s$ to $G_i$, thereby providing a direct feedback inhibition of the AC signalling (that initiated the process) and initiating a second signalling pathway resulting in the activation of MAP kinases [276]. Activation of MAP kinases, specifically of the extracellular signal-regulated kinases 1 and 2 (ERK 1/2) is now recognized to be a major signal transduction pathway of many G protein-coupled receptors. In contrast to the observations of G-protein switching, others have shown in HEK293 cells that the activation of the MAP kinase pathway by $\beta_2$-adrenoceptors, although PKA-dependent, was pertussis toxin insensitive, indicating that $\beta_2$-adrenoceptors do not need to switch to $G_i$ [277]. Moreover, additional studies with a PKA-insensitive mutant of the $\beta_2$-adrenoceptor revealed that $\beta_2$-adrenoceptor activation of ERK1/2 does not require PKA phosphorylation of the $\beta_2$-adrenoceptor receptor and subsequent switching from activation of $G_s$ to $G_i$ [278]. Evidence has emerged that ERK1/2 activation of $\beta_2$-adrenoceptors may be mediated by a family of cAMP-binding proteins termed cAMP-GEFs [279] or Epacs [280]. These proteins show increased GEF activity towards Rap 1, which may activate ERK1/2 through B-raf, upon cAMP binding [281,282]. Additionally, it has been reported in HEK-293 cells that ERK activation by the $\beta_2$-adrenoceptor, mediated by these Epac proteins, may lead via Rap2B and PLC\textsubscript{C} stimulation to an increase in intracellular calcium, finally activating H-Ras as a trigger of the MAP kinase cascade [283].
Functional antagonism between β₂-adrenoceptor and contractile stimuli

One of the mechanisms that may contribute to the functional antagonism between contractile and relaxing stimuli could be a cAMP-dependent inhibition of the inositol phosphate (IP) response. Both in canine [266] and bovine [284-286] tracheal smooth muscle, it has been demonstrated that β₂-adrenoceptor agonists and other cAMP elevating agents such as forskolin and dibutryl cyclic AMP attenuate histamine-induced IP formation. In contrast, no such effect is observed with respect to the phosphoinositide hydrolysis induced by the full muscarinic agonists carbachol or methacholine, whereas the partial muscarinic agonists pilocarpine and McN-A-343, but not oxotremorine, were susceptible to such an inhibition [266,286]. It could be imagined that McN-A-343 and pilocarpine exert their effects through a different stimulation pattern of muscarinic receptor subtypes, compared to oxotremorine and the full agonists methacholine and carbachol, as both M₂ and M₃ receptors are present in bovine tracheal smooth muscle [243]. Although methacholine-induced phosphoinositide hydrolysis and contraction are mediated by the M₃-receptor [121] M₂-receptors, which are negatively coupled to AC, represent the major (70-80%) proportion of the population. The resistance of oxotremorine-, methacholine- and carbachol-induced phosphoinositide metabolism to agonists that stimulate cAMP synthesis could therefore relate to their ability to inhibit AC via stimulation of muscarinic M₂-receptors. However, this explanation is rather unlikely as methacholine-induced IP formation was also resistant to cAMP elevations induced by forskolin and stable analogues of cAMP [266]. In addition, in guinea pig [287], bovine [288] and human [289] airway smooth muscle no role for M₂-receptors in the functional antagonism between contractile and relaxant stimuli was observed. Interestingly, muscarinic agonist-induced contraction in airway smooth muscle is also relatively resistant to relaxation by β₂-adrenoceptor agonists, compared with contractions induced by histamine [290-292], suggesting that this resistance is related to the lack of effect of the β-agonists on muscarinic agonist-induced IP formation. However, in bovine tracheal smooth muscle cells it has been demonstrated that cAMP dependent inhibition of contractile agonist-induced Ca²⁺-responses is not primarily caused by attenuation of the IP production and that the relative resistance of muscarinic agonist-induced contraction to β₂-adrenoceptor agonists resides in its higher potency in inducing IP formation and subsequent intracellular Ca²⁺-changes [158]. In fact, a striking correlation was found between isoprenaline-induced relaxation of bovine tracheal smooth muscle contraction and inhibition of Ca²⁺-mobilization or influx, indicating that not IP₃ formation but the subsequent changes of calcium homeostasis play the major role in β₂-adrenoceptor-mediated relaxation of methacholine- and histamine-induced contraction. A possible mechanism reducing contractile agonist-induced Ca²⁺-mobilization could be a cAMP-dependent inhibition of IP₃-binding to the IP₃-receptor. IP₃-receptors have been identified as substrates for PKA [288,293], and PKA-mediated phosphorylation of the IP₃-receptor markedly reduces the ability to release Ca²⁺ from intracellular stores [293,294]. Indeed, using competitive binding assays it has been demonstrated in rabbit tracheal smooth
membranes that activation of the cAMP-signalling pathway inhibits IP₃ binding to the receptor [295].

**Regulation of the β₂-adrenoceptor**

*Classification of desensitization*

The term desensitization refers to any pathway that results in the loss of responsiveness of a receptor signalling system. Two basic classes of desensitization have been distinguished, namely homologous and heterologous desensitization [296]. The term homologous (or receptor-specific) desensitization indicates that when a receptor is activated by an agonist, only this receptor is becoming refractory to subsequent agonist application, without affecting other receptors or receptor systems present in the same cell. In contrast, heterologous (or receptor-nonspecific) desensitization indicates that stimulation of one receptor attenuates the response to multiple (distinct) receptors operating through the same or different signalling pathways. In contrast to homologous desensitization, agonist binding to the receptor is not necessary for heterologous desensitization. Both mechanisms have been studied extensively for the β₂-adrenoceptor [297-299] which has often been used as a model for the regulation of G protein-coupled receptors. Phosphorylation appears to be an essential step both for homologous and heterologous desensitization, but the mechanisms are quite distinct.

*Homologous desensitization*

Homologous desensitization of the β₂-adrenoceptor is primarily mediated by activation of G-protein-coupled receptor kinases (GRKs). GRKs phosphorylate the agonist-occupied form of the receptor, after translocation to the plasma membrane by anchoring to free Gβγ subunits generated upon receptor activation [300], but slightly uncouple the receptor from Gα. GRK-mediated phosphorylation also promotes binding of β-arrestins to the receptor, resulting in a much more effective uncoupling of the receptor from its stimulatory G-protein by sterically interfering with the receptor-Gα interaction and thus terminating the β₂-adrenoceptor signalling [112,256,297,301,302]. The role of GRK-mediated phosphorylation is to increase the affinity of the receptor for arrestins. *In vitro*, the β-arrestin-1-binding affinity of β₂-adrenoceptors is increased 10- to 30-fold following phosphorylation of the receptor by GRK [303]. It is the binding of arrestin to the receptor, rather than GRK-mediated phosphorylation, that leads to homologous desensitization of the receptor. GRK-mediated arrestin binding is followed by sequestration and downregulation of the receptor, which occurs via the association of the receptor-arrestin complex with components of clathrin-coated pits [304-306]. Only the receptor that is occupied by the agonist is in the appropriate conformational state to be a suitable substrate for GRKs. This strict agonist-binding requirement for GRK-mediated phosphorylation makes this mechanism homologous in that only the simulated receptor is being desensitized.
The GRK family of serine/threonine kinases consists of seven isoforms that share a number of structural and functional similarities and GRKs 1-6 have been shown to phosphorylate the β2-adrenoceptor in its C-terminal cytoplasmatic tail [299,307]. Thus far, GRK2 (also referred to as βARK1 (β-adrenergic receptor kinase-1)) is the major kinase implicated in homologous desensitization of the β2-adrenoceptor. For example, reconstitution of β2-adrenoceptors and highly purified GRK2 along with β-arrestin in vitro was sufficient to establish maximal β2-adrenoceptor desensitization [302], and overexpression of GRK2 in CHO cells enhanced desensitization of the β2-adrenoceptor to high concentrations of agonist [308]. However, a number of studies suggest additional roles for GRK3 (βARK2) and GRK5 in β2-adrenoceptor desensitization [306,309-311]. GRK2 and 3 are rapidly translocated from the cytosol to the plasma membrane after agonist stimulation [312,313]. They contain a 125-residue extension at the C-terminus that interacts with isoprenylated βγ subunits of trimeric G-proteins [300,309,313-317]. Free βγ subunits are only found in the plasma membrane at sites of receptor activation, so that this is an extremely precise mechanism for targeting these kinases to activated receptors. In contrast, most GRK5 is normally present at the cell membrane, and there appears to be a direct interaction of the C-terminal domain with phospholipid-head groups of the membrane [318]. Autophosphorylation of GRK5 is required for receptor-phosphorylating activity [319]. Interestingly, intracellular calcium levels may regulate homologous desensitization of β2-adrenoceptors as it has been demonstrated that all three GRKs are inhibited by ubiquitously expressed calmodulin in a calcium-dependent fashion [320-323].

 Arrestins not only have an important role in uncoupling β2-adrenoceptors from their signalling pathway, but they are also crucial mediators of receptor sequestration or internalization following prolonged agonist exposure [301,324-328]. Specifically, β-arrestins function as adaptor scaffolding proteins that link β2-adrenoceptors to components of the endocytic machinery, including clathrin and β2-adaptin (the β-subunit of the adaptor protein AP2) [329-331]. In human astrocytoma cells it was found that translocation of β2-adrenoceptors from the plasma membrane to an intracellular compartment upon isoprenaline stimulation occurred very rapidly, exhibiting a t1/2 of ~2 min after a delay of ~1 min [332]. This sequestration process has originally been considered to be another mechanism of desensitization. However, the majority of sequestered receptors are already desensitized as the consequence of phosphorylation. In addition, in human astrocytoma cells, rapid β2-adrenoceptor desensitization could be observed prior to any decrease in cell number [332]. Moreover, inhibiting β2-adrenoceptor sequestration through clathrin coated pits did not inhibit its rapid agonist-induced desensitization [333]. Studies have now suggested that the major role of sequestration in short-term regulation of the receptor may be in resensitization, since it appears that the sequestered pool is the site of dephosphorylation of the receptor [324,326,327]. Thus, internalized β2-adrenoceptors can be dephosphorylated in endosomes, enriched with specific phosphatases, and subsequently recycled back to the cell surface as fully functional resensitized receptors [334,335]. Direct evidence for this hypothesis came from the observation that inhibition of β2-adrenoceptor internalization through clathrin-coated pits with either concanavalin A or sucrose prevented
resensitization [333,336]. More recently, a G protein-coupled receptor phosphatase (GRP) was identified, a member of the PP2A family, which was colocalized with the β2-adrenoceptor in endosomes during agonist-promoted receptor internalization. This phosphatase dephosphorylates the receptors in acidic environments such as those found in endosomes, and it has been suggested that the low intraluminal pH of the endosomes induces conformational changes in the receptor that can be recognized by GRP [337]. Interestingly, sequestration does not occur under conditions that specifically induce heterologous desensitization (discussed below).

**Figure 2** Upon ligand binding, the β2-adrenoceptor activates adenylyl cyclase through coupling to heterotrimeric Gs protein, leading to cAMP synthesis (step 1). GRK2 is recruited to the ligand-bound β2-adrenoceptor and phosphorylates the receptor (step 2). Once phosphorylated, the receptor binds to β-arrestins, which sterically hinders further coupling of the receptor to G-proteins (step 3). The β-arrestin, through its binding to clathrin and AP2 protein, targets the phosphorylated receptor to clathrin-coated pits and promotes receptor internalization (step 4). Following its dephosphorylation by a protein phosphatase (step 5), the receptor is recycled back to the cell surface (step 6) or is degraded in the lysosomes (step 7). Figure adapted from Métayé et al. [338].

**Heterologous desensitization**

In addition to GRKs, phosphorylation of the β2-adrenoceptor by second-messenger kinases such as PKA and PKC may also contribute to desensitization by uncoupling the receptor from Gs. However, this uncoupling does not need β-arrestin binding and is not associated with subsequent sequestration of the receptor. Agonist occupancy of the target β2-adrenoceptor is not required for this process, thus receptors that have not bound to an
agonist, including receptors for other ligands, can be desensitized by the activation of PKA or PKC. This process is therefore termed heterologous desensitization. As described before, PKA is activated through the formation of cAMP upon β2-adrenoceptor stimulation and a variety of studies have implicated a role for PKA in β2-adrenoceptor regulation. β2-Adrenoceptors in turkey erythrocytes [339], as well as in hamster lung, were shown to be phosphorylated by PKA and this phosphorylation directly reduced β2-adrenoceptor interaction with Gi [340]. Site-directed mutagenesis studies have indicated that PKA phosphorylates the serine/threonine residues in the third intracellular loop of the receptor and the C-terminal cytoplasmatic tail [174, 341]. However, only phosphorylation at the third intracellular loop is required for PKA-dependent desensitization [342]. Thus, cells expressing β2-adrenoceptors lacking this site, were unable to undergo desensitization following exposure to adrenaline, whereas cells lacking the site on the C-terminal cytoplasmatic tail exhibited β2-adrenoceptor desensitization comparable to that observed in cells expressing the wild type β2-adrenoceptor. Interestingly, it has been demonstrated that PKA phosphorylation of the β2-adrenoceptor involves the role of A-kinase anchoring proteins (AKAPs). AKAPs are a family of structural diverse proteins that compartmentalize PKA to specific target organelles via interaction with the RII regulatory subunit of PKA [343]. The first indication of the role of AKAPs in β2-adrenoceptor function was provided by the demonstration that AKAP250 (also termed gravin) interacts with the carboxyl terminal tail of the β2-adrenoceptor, which has been implicated in receptor phosphorylation, desensitization, internalization and resensitization [344-346]. The proposed model involves a constitutive binding of AKAP250 to the β2-adrenoceptor, not only regulating receptor phosphorylation (via PKA or possibly PKC) and dephosphorylation (via protein phosphatase 2B) but also contributing to the incorporation of additional proteins such as GRKs and arrestins, into the complex [345]. In addition, AKAP79/150 also appears to constitutively associated with the β2-adrenoceptor, resulting in a complex that contains PKA, PKC and protein phosphatases 2B. Indeed, it has been found that AKAP79/250 promotes β2-adrenoceptor phosphorylation after agonist stimulation and is even able to facilitate activation of MAPK pathways [345].

Another important kinase involved in heterologous desensitization of the β2-adrenoceptor is PKC. As discussed before, PKC is activated upon stimulation of Gq-coupled receptors. PKC is able to phosphorylate the β2-adrenoceptor at sites in the third intracellular loop, leading to uncoupling of the receptor [347-349]. In airway smooth muscle, much attention has been paid to this mechanism of receptor cross-talk as it was observed that contractions induced by muscarinic agonists are relatively resistant to relaxation by β2-agonists compared with contractions induced by histamine. Thus, in canine and bovine tracheal smooth muscle it has been demonstrated that β2-adrenoceptor-mediated and non receptor-mediated elevation of cAMP potently inhibited histamine- but not methacholine-induced accumulation of inositol phosphates [266, 285]. Furthermore, several studies have shown that exaggerated cholinergic stimulation of airway smooth muscle causes a reduced relaxability of the muscle by β2-adrenoceptor agonists. In human [122, 350] and animal [123, 290, 291, 351-353] airway smooth muscle, both the potency and the maximal relaxation
are gradually reduced in the presence of increasing concentrations of contractile agonists. In addition, such diminished functional antagonism has also been observed in vivo [292]. Interestingly, the responsiveness of the β2-adrenoceptor in the presence of contractile agonists is not determined by the contraction level only, but also by the agonist under investigation [290-292,351]. Furthermore, in guinea pig tracheal smooth muscle it was found that the reduction of the isoprenaline-induced relaxation was relatively large for methacholine, intermediate for oxotremorine and histamine, and small for the partial muscarinic agonist McN-A343 [291]. Since the efficacy of the contractile agonist to induce IP production showed a striking correlation with their capacity to reduce isoprenaline-induced relaxation, the possibility was considered that direct interference by phosphoinositide metabolism of β2-adrenoceptor function may play an important role, possibly through DAG-induced PKC activation. Indeed, in various cells and tissues it has been found that activation of PKC via agonist-induced PI metabolism or phorbol esters is able to desensitize the β2-adrenoceptor, presumably via phosphorylation of the receptor and/or Gs [354-359]. Biochemical evidence that cross-talk between PI metabolism and β2-adrenoceptors could play a role in the regulation of the responsiveness of β2-adrenoceptors of airway smooth muscle by contractile agonists was obtained in bovine tracheal smooth muscle, showing that incubation with the phorbol ester PMA and carbachol resulted in β2-adrenoceptor uncoupling and a reduced β-agonist-induced cAMP response [360].

Relative contributions of PKA and GRKs in rapid β2-adrenoceptor desensitization

The relative contributions of PKA and GRKs to the overall manifestation of desensitization of β2-adrenoceptors following β-agonist stimulation is complex. Using isoprenaline, it has been established in permeabilized A431 cells, that under conditions of selective blockade of PKA (with PKI, an inhibitory peptide) or GRK2 (by heparin) still significant desensitization was observed, indicating that both desensitization pathways are indeed functionally operative. However, at low receptor occupancy (agonist concentration <10 nM) the PKA pathway was selectively activated [361]. This observation was confirmed by studies using mutant β2-adrenoceptors lacking putative GRK2 and PKA-phosphorylation sites [362,363], with the capacity for both GRK2- and PKA-mediated desensitization with high agonist concentrations. Using both heparin and PKI it was demonstrated in permeabilized A431 cells that isoprenaline-induced phosphorylation of the β2-adrenoceptor by GRK2 occurred with a t1/2 of <20 seconds, whereas phosphorylation by PKA had a t1/2 of about 2 min [364]. Similarly, GRK2-mediated desensitization of the β2-adrenoceptor proceeded with a t1/2 of <15 sec, and PKA-mediated desensitization with a t1/2 of about 3.5 min. Maximal desensitization, mediated by the two kinases corresponded to a reduction of the signal-transduction capacity of the receptor/AC system by about 60% in the case of GRK2 and by about 40% in the case of PKA. These findings indicate that GRK2-mediated phosphorylation is the most rapid and quantitatively most important factor contributing to the rapid desensitization [364] and are consistent with other observations in desensitization studies where heterologous desensitization of the β2-adrenoceptor typically is observed.
later and of a lesser magnitude when compared to homologous desensitization [365]. In addition, a number of studies have confirmed that with higher agonist concentrations (>50 nM), GRK-induced desensitization is quantitatively more important [299,366,367]. Evidence for acute β2-adrenoceptor desensitization in cultured human airway smooth muscle cells was first demonstrated by Hall and colleagues [368], showing that prior treatment of the cells with varying concentrations of isoprenaline for 1 to 16 hours produced concentration-related desensitization of cAMP responses to subsequent challenge with isoprenaline, ranging from 62 to 85% desensitization. It was also observed that exposure to PGE2 produced a concentration-related desensitization of cAMP responses to subsequent challenge with isoprenaline. Acute regulation of the β2-adrenoceptor in human airway smooth muscle was further characterised by Penn and colleagues [256], to elucidate mechanisms by which desensitization of β2-adrenoceptors occurs in airway smooth muscle. A 30 min pretreatment of the cells, with either β-agonist (isoprenaline) or with agents that increased intracellular cAMP response independently of β2-adrenoceptor activation (forskolin and PGE2), resulted in a loss of β2-adrenoceptor responsiveness to subsequent isoprenaline challenge, characterised by a diminished cAMP response. No significant changes were measured in forskoline-induced cAMP production, indicating that the β2-adrenoceptor is the principle locus of regulation. Isoprenaline-induced desensitization was characterised by an ~60% loss of maximal responsiveness to isoprenaline, which was only minimally but significantly prevented by PKA-inhibition, indicating a quantitatively more important role for GRK-induced desensitization. In addition, overexpression of GRK2 in human airway smooth muscle cells enhanced isoprenaline-induced desensitization, implicating that GRK2 is indeed a mediator of β-agonist induced desensitization in human airway smooth muscle. Furthermore, isoprenaline-induced desensitization was associated with, but not dependent on a ~45% loss of cell surface β2-adrenoceptors, and rapid recovery from isoprenaline-induced desensitization was highly dependent on sequestration. In contrast, β2-adrenoceptor desensitization induced by forskolin and PGE2 was characterized by only ~20-30% loss of maximal responsiveness, which was largely prevented when PKA was inhibited, indicating a primary role for PKA in this form of heterologous β2-adrenoceptor desensitization [256].

**Heterologous regulation of homologous desensitization**

Interestingly, heterologous and homologous desensitization are not necessarily completely independent processes. In recent years, phosphorylation of GRKs at different sites and by a variety of protein kinases has emerged as an important mechanism for regulation of their activity, interaction with other proteins and even protein stability. In addition to the direct effects on receptor/G-protein coupling, PKC has been shown to increase GRK2 activity and enhance membrane targeting. The capacity of PKC to activate GRK2 was originally suggested in studies in human mononuclear leukocytes in which calcium iononophores or phorbol esters caused an increase in cytosolic GRK2 activity that was blocked by prior cell treatment with PKC inhibitors [369]. GRK2 was shown to be a substrate for PKC both in
vitro and in intact cells, and functional significance of the PKC-dependent increase in GRK2 activity was demonstrated by β-adrenoceptor homologous desensitization experiments in intact mononuclear leukocytes. Thus, β-adrenoceptor desensitization, induced by exposure of the cells to 10 μM isoprenaline for 5 min, was markedly increased in PMA-pretreated cells and this increase was inhibited by the GRK2 inhibitor heparin. In an additional study it has been demonstrated that PKC-induced phosphorylation of GRK2 enhances its activation by translocating GRK2 to the cell membrane [370]. Furthermore, using human mononuclear leukocytes, it was shown that T-cell activation resulted in increases in GRK2 mRNA, which could be mimicked by the combined application of calcium ionophore and low doses of the PKC activator PMA, indicating that PKC is also involved in increasing GRK2 mRNA expression [371].

In both in vitro and intact transfected COS-1 cell studies, PKC was also shown to phosphorylate GRK5 at two sites within the C-terminal 26 acids [320]. In contrast to the observed activation of GRK2, GRK5 phosphorylation by PKC dramatically reduced GRK5 activity, demonstrated by the reduced binding of GRK5 to rhodopsin containing membranes and reduced phosphorylation of the light-activated rhodopsin receptor. In addition, PKC-mediated phosphorylation significantly reduced GRK5 catalytic activity towards non receptor substrates. As discussed before, both GRK2 and GRK5 are inhibited by Ca2+/calmodulin. Together with the demonstrated effects of PKC on GRK-activity, these findings suggest a coordinated regulation of G protein-coupled receptors when both PKC and Ca2+/calmodulin are activated by Gq-coupled receptors. Interestingly, it has recently been demonstrated in vitro that the tonic inhibition of GRK2 by Ca2+/calmodulin was almost completely abolished when GRK2 was phosphorylated by PKC, indicating another possible mechanism for activation of GRK2 [372].

In addition to PKC, PKA has also been shown to directly phosphorylate GRK2, leading to an enhanced GRK2 activity towards β2-adrenoceptors [373]. In fact, PKA-mediated phosphorylation does not actually affect the kinase activity, but rather enhances binding of GRK2 to Gβγ subunits, thereby facilitating membrane targeting of GRK2 and interaction with activated receptors. However, the ability of PKA to phosphorylate GRK2 is dependent on the presence of Gβγ-bound to GRK2 and on PKA being tethered to the receptor by AKAP79 [373]. Thus, PKA-induced GRK2-phosphorylation increased its binding affinity for Gβγ subunits and thereby promoted the recruitment of GRK2 to the plasma membrane and into a complex with its activated receptor substrates.

**Downregulation**

Finally, β2-adrenoceptors may also undergo receptor downregulation. Downregulation is defined as a loss in receptor density and occurs as a result of increased receptor degradation or reduced receptor synthesis. As opposed to the relatively rapid desensitization processes of receptor phosphorylation, uncoupling and sequestration, receptor downregulation represents a chronic adaptation that occurs with prolonged agonist exposure of the cell.
Downregulation of the β₂-adrenoceptor does not appear to require receptor phosphorylation since several studies have demonstrated that mutant β₂-adrenoceptors containing altered phosphorylation sites have normal patterns of downregulation \[362,374,375\]. However, mutagenesis of the PKA phosphorylation site of the β₂-adrenoceptor decreased the rate and extent of the agonist-induced downregulation, indicating that PKA may play a facilitatory role \[341\]. A possible mechanism of β₂-adrenoceptor down-regulation may involve reduction in β₂-adrenoceptor synthesis as a result of cAMP-mediated destabilization of β₂-adrenoceptor mRNA, as it was observed in DDT₁MF-2 cells that chronic exposure to isoprenaline resulted in a time-dependent reduction in β₂-adrenoceptor mRNA levels due to a reduction in mRNA stability \[376-378\]. Enhanced degradation of the PKA-phosphorylated β₂-adrenoceptor could be an alternative explanation for PKA-induced downregulation \[341\]. In guinea pig lung there is a marked reduction in β₂-adrenoceptor density after a 7 days infusion of noradrenaline, which is accompanied by a similar reduction in steady state β₂-adrenoceptor mRNA levels, although the effect in airway smooth muscle was less pronounced than that observed in lung parenchyma \[379\]. Similar data were obtained in rats after prolonged infusion with isoprenaline \[380\]. In addition to the reduction in β₂-adrenoceptor mRNA, there is also a reduction in the activity of the transcription factor CREB, which may account for the reduced rate of β₂-adrenoceptor gene transcription. In contrast, as described before, short-term agonist stimulation of the β₂-adrenoceptor results in a transient increase in receptor mRNA levels through a direct increase in the rate of β₂-adrenoceptor gene transcription, by enhancing the activity of CREB \[269,271\]. However, the discovery of the CRE modulator (CREM), a CREB-related transcription factor which binds CREs in a dominant-negative fashion, suggests that under certain circumstances cAMP generation could also result in downregulation of β₂-adrenoceptor gene transcription \[381\].
β₂-Adrenoceptor function in asthma

Responsiveness of the β₂-adrenoceptor system

Endogenous β-agonists

The extent of sympathetic innervation to the lung is species dependent [382]. Cats have extensive sympathetic innervation in the lung including the innervation of the airway smooth muscle [383]. In guinea pig airways, main sympathetic projections are to blood vessels, the bronchi being innervated only sparcely [254]. In humans, sympathetic fibers innervate the submucosal mucus gland, blood vessels, and the parasympathetic ganglia, but do not directly innervate airway smooth muscle [384-387]. In line with the lack of direct sympathetic innervation of airway smooth muscle, β₂-adrenoceptors, but not β₁-adrenoceptors, are present throughout the human lung [248,250-252,254,385,386] and autoradiographic and radioligand binding studies have shown that β₂-adrenoceptor density increases with increasing airway generation [246,251]. In addition, relaxation of human central and peripheral airway smooth muscle has shown to be mediated by a homogenous population of β₂-adrenoceptors [253,254] and control of airway smooth muscle tone is mainly through circulating adrenaline [388], which is secreted from the adrenal medulla. The other endogenous catecholamine, noradrenaline, is the neurotransmitter and only appears in the plasma by overflow from sympathetic nerve activity. In patients with asthma, β-adrenoceptor blockade is known to induce bronchospasm and aggravation of the disease [389-392], suggesting that endogenous bronchodilating β-adrenergic activity is important in controlling airway smooth muscle tone in asthmatic patients. Even low systemic doses of β-blocking eye drops may cause wheeziness in asthmatics [393] and it has been demonstrated that β-receptor-blockade of asthmatics causes immediate bronchoconstriction and increases bronchial responsiveness to methacholine and histamine [394,395]. In contrast, in non-asthmatics large doses of beta-blocking drugs have little or no effect on airway function and responsiveness [396,397], suggesting a more important role for circulating adrenaline in patients with asthma. Although venous plasma levels of adrenaline have been demonstrated to be normal in asthmatic subjects [398-401], a temporal relationship has been observed between decreases in plasma adrenaline and decreases in peak expiratory flow rate during the early morning hours in patients with nocturnal asthma [402,403]. In addition to asthmatic patients, there is also a chronobiological rhythm in catecholamine secretion in normal subjects, being lowest between 1 and 5 a.m [404]. The fall in endogenous adrenaline level at night seems to be due to reduced secretion from the adrenal medulla, since plasma clearance is unchanged [405]. The reduced adrenaline level at night closely correlate with the decrease in peak flow, suggesting that decrease of the protective influence of adrenaline may be the cause of bronchoconstriction at night, in the same way that β-adrenoceptor antagonists produce bronchoconstriction in asthmatics, by blocking the effect of endogenous adrenaline.
As described before, adrenaline may produce bronchodilatation directly by stimulating $\beta_2$-adrenoceptors on airway smooth muscle, and indirectly by inhibiting the release of inflammatory mediators from pulmonary mast cells and by modulation of cholinergic neurotransmission. Thus, the reduced circulating adrenaline concentrations at night may lead to bronchoconstriction by a permissive effect on pulmonary mast cell mediator release. In support of this, plasma histamine concentrations, used as an indicator of mediator secretion, rise at night in asthmatic patients, and the plasma histamine concentration is inversely correlated with the peak flow and plasma adrenaline level. Infusion of adrenaline in a low concentration to reverse the fall in endogenous adrenaline reduces the elevated plasma histamine concentration at night [402]. In normal subjects, no significant variations in plasma histamine concentration are seen, supporting the idea that the increased secretion of bronchoconstrictor mediators from the sensitized mast cells of asthmatic airways amplify the nocturnal changes in airway tone.

An important regulator of the actions of circulating adrenaline at the airway smooth muscle site is catechol-O-methyltransferase (COMT). Circulating adrenaline, but also other neurotransmitters and drugs with a catechol structure, are rapidly inactivated by COMT. Positron emission tomography studies have demonstrated high COMT activity in kidney, liver, intestine, stomach, spleen, lungs and heart of mice [406]. In addition, rather high COMT activities have been described in human lung [407] and rat lung [408]. COMT transfers a methyl group from 5-adenyl methionine to the 3-OH position of the catecholamine. As a consequence, the conjugated compound has no adrenergic activity and may be either excreted or further metabolized by monoamine oxidase [409]. Indeed, it has been demonstrated that $3H$-noradrenaline in isolated rat lungs [408] and $3H$-isoprenaline in isolated guinea pig tracheal rings [410] and rabbit tracheal smooth muscle [411] is O-methylated, which could be prevented by COMT-inhibition. Interestingly, in guinea pigs the O-methylation activity of the “smooth muscle-rich” component of the trachea was approximately three-times higher than for complete tracheal rings [410]. Various functional studies in airway smooth muscle have indicated that COMT-activity is counteracting catecholamine-induced smooth muscle relaxation. Thus, in pig bronchus the COMT-inhibitor U-0521 caused a 6 fold increase in potency of isoprenaline-induced relaxation [412] and the potency of isoprenaline was increased 7 fold in the presence of U-0521 in guinea pig tracheal smooth muscle rings after 50% carbachol contraction with no effect on maximum relaxation [413]. Based on the evidence presented above, COMT-activity seems to play an important role in counteracting the relaxing ability of endogenous and exogenous catecholamines and therefore may be involved in creating a disbalance between contractile and relaxing pathways in patients with asthma. In support of this hypothesis it is interestingly to know that increased activation of COMT has been found in children with asthma [414].
Possible cross-talk between PI metabolism and the β₂-adrenoceptor system

As discussed before, inhaled β₂-adrenoceptor agonists are effective bronchodilators and widely used to control airway function in asthma and chronic obstructive pulmonary disease (COPD) [415,416]. The efficacy of these drugs in asthma is mainly due to the functional antagonism counteracting the bronchoconstrictor effects of neurotransmitters and mediators released in airway inflammation [97]. However, it is well known that patients during a severe exacerbation of asthma have a reduced bronchodilator response to β₂-adrenoceptor agonists [417]. In addition, studying β₂-adrenoceptor abnormalities in leukocytes of non-treated asthmatic patients, it was demonstrated that a loss in β₂-adrenoceptor function indeed may occur, predominantly in those with active and severe asthma [418-421]. Evidence has emerged that the described mechanisms of cross-talk between PI metabolism and the β₂-adrenoceptor system may play a role in asthmatic airways. In isolated preparations of patients with fatal [422-424] or non-fatal [251,425] active and severe asthma, the relaxant response to isoprenaline is reduced, whereas a normal response has been found in patients with mild to moderate asthma [426,427]. Importantly, the reduced β₂-adrenoceptor relaxation was found in these studies irrespective of prior use of β-agonists. Evidence for β₂-adrenoceptor uncoupling from Gₛ has been suggested in autoradiographic and functional studies in lungs and bronchial preparations from patients with fatal and non-fatal asthma, since normal [428] or even enhanced [251,429] β₂-adrenoceptor densities were found in airway smooth muscle, while the β₂-adrenoceptor relaxation was reduced. These results indicate that the β₂-adrenoceptor is uncoupled from its effector system in patients with severe asthma. In addition, in lymphocytes from asthmatic patients it has been found that the β₂-adrenoceptor cAMP response was reduced after allergen challenge [392,430]. Since PKC activation, via receptor-mediated stimulation of PI metabolism, is involved in the activation of various cells involved in the allergic response, including airway smooth muscle and inflammatory cells, it can be imagined that PKC-induced (heterologous) desensitization could cause a reduced β₂-adrenoceptor responsiveness of these cells and thus contribute to enhanced airway reactivity in patients with active and severe asthma. Indeed, using a guinea pig model of allergic asthma, it has been found that single or repeated allergen challenge may lead to a reduced isoprenaline-induced relaxation of methacholine- and histamine-contracted preparations [431]. As the reduced β₂-adrenoceptor sensitivity in vitro was paralleled by a progressive infiltration of inflammatory cells in the airways, it was suggested that mediators from these cells may decrease airway smooth muscle β₂-adrenoceptor sensitivity. PKC seems to be the most likely candidate involved in this process (see paragraph ‘β₂-adrenoceptor function in asthma’).
General introduction

Adverse effects of β2-adrenoceptor agonist therapy in asthma

Regular treatment with β2-agonists

Almost since the use of inhaled β-agonists in asthma started, there has been a concern that regular use or overuse may increase asthma morbidity and mortality [432]. This was largely initiated by the epidemics of asthma deaths following the introduction of high-dose isoprenaline in the UK and other countries in the 1960s and following the introduction of fenoterol in New Zealand in the late 1970s [433]. In case-control studies the risk of death or near-death from asthma was shown to be significantly greater in patients prescribed inhaled fenoterol [434]. It has also been found that asthma control was worse in patients receiving regular inhaled fenoterol for 24 weeks than when they used the bronchodilator “as needed” [85], thus questioning the safety of the short acting β-agonist fenoterol. Meanwhile, increases in asthma morbidity and mortality were also occurring in countries in which fenoterol was not widely prescribed, but where there was a progressive rise in the overall use of inhaled β-agonists [435], suggesting that the increased use of relatively high doses of β-agonists as a class and not just fenoterol might cause these adverse epidemiological trends. This suggestion was supported by epidemiological surveys which detected an association between the intensity of inhaled β-agonist drug therapy and asthma death or near death [86,436]. Furthermore, 1 year of regular inhalation of salbutamol by patients with asthma and COPD increased bronchial responsiveness to histamine [437], while during a 2-year treatment period a rapid decline in lung function was found compared to symptomatic use of salbutamol [438]. In addition, treatment of patients with mild to moderate persistent asthma with long acting β-agonists induced deterioration of lung function, bronchial hyperresponsiveness, as well as an increased number of exacerbations [439-441]. Moreover, rebound increase in bronchial reactivity [442,443], hyperreactivity to histamine and methacholine [444-446] and increased bronchial responses to allergens [447-449] were reported after discontinuation of regular treatment with β-agonists.

A well-recognized effect of the regular administration of β-agonists is the development of tolerance, which underlie the worsening of asthma following long-term treatment with β-agonists. For both short- and long-acting β-agonists decreased bronchodilator responses have been demonstrated in asthmatic patients treated on a regular basis [450-458]; however the outcome differed among these studies [459-464] and some studies even showed no tendency to develop tolerance for formoterol [465-467] and salmeterol [468,469]. Several studies have examined the effect of chronic exposure to β-agonists on bronchodilator and bronchoprotective responses. Remarkably, a dissociation has been observed in the efficacy of β-agonists to induce bronchodilatation in asthmatic patients after prolonged treatment with these agonists, and changes in their capacity to protect against bronchoconstrictor stimuli. Thus, regular use of several β-agonists caused a significant reduced protection against methacholine- [449,461-463] and/or adenosine monophosphate (AMP) [461,464]-induced bronchoconstriction, with no effect on the bronchodilator effect of these β-agonists.
A possible explanation for the dissociation in desensitization susceptibility between the bronchodilator and bronchoprotective effects might be that most studies investigating the bronchodilator response have been performed in patients with mild to moderate asthma with a good reversibility of airway obstruction. Therefore, it could be suggested that more severe bronchoconstriction induced by contractile agonists may be necessary to reveal reduced functional antagonism upon \( \beta_2 \)-adrenoceptor desensitization [470]. Interestingly, when AMP was used as the provocation stimulus, loss of protection was significantly more pronounced compared to methacholine [461]. Since AMP is thought to produce bronchoconstriction indirectly by releasing mediators from airway mast cells, this finding suggests that tolerance of the mast cell-stabilizing effects of \( \beta \)-agonists may be important for the deterioration of asthma symptoms and bronchial hyperreactivity after long-term treatment and that \( \beta_2 \)-adrenoceptors on mast cells may be more susceptible to tolerance than those on airway smooth muscle. Evidence for this hypothesis was found in sensitized guinea pigs showing that \( \beta_2 \)-adrenoceptors mediating inhibition of allergen-induced mediator release were more susceptible to desensitization than \( \beta_2 \)-adrenoceptor mediating airway smooth muscle relaxation [471].

An alternative mechanism was recently suggested by McGraw and colleagues [472], whereby chronic exposure to \( \beta \)-agonists might alter airway responses. Using different mouse models to examine \( \beta_2 \)-adrenoceptor function, they found that \( \beta_2 \)-adrenoceptor knockout mice have reduced responses to bronchoconstrictor stimuli compared with wild-type mice, whereas mice overexpressing the \( \beta_2 \)-adrenoceptor have increased bronchoconstrictor responses. This increase in bronchoconstrictor responses in mice overexpressing the \( \beta_2 \)-adrenoceptor was associated with an increased expression of the relevant isoform of PLC (PLC\( _\beta \)), which was thought to account for the enhanced response to contractile agents in this system. If such mechanism is also present in human airways, one might predict that chronic exposure of airway smooth muscle to \( \beta_2 \)-adrenoceptor stimulation might also lead to increased expression of PLC\( _\beta \) and hence increased contractile responses.

**Enantiomers of \( \beta \)-agonists**

A number of possible mechanisms have been discussed above to explain the adverse effects of chronic \( \beta \)-agonist therapy. Another explanation for the detrimental effects of \( \beta \)-agonists may involve their racemic nature. Although endogenous adrenaline, which is importantly involved in bronchodilatation and suppression of mast cell activation, is always in the single R-enantiomer form, all marketed selective \( \beta \)-agonists are racemic, composed of equal amounts of the R-enantiomer and its nonsuperimposable mirror image, the S-enantiomer. The R-enantiomer of \( \beta \)-agonists is thought to exhibit the observed bronchodilatation and clinical benefit of the racemate [473], whereas the S-enantiomer is devoid of clinical benefit and is assumed to be virtually inert. In recent years, the suspicion has been raised that the S-enantiomer of \( \beta \)-agonists is responsible for induction of airway hyperreactivity and may contribute to increased asthma morbidity and mortality [474-478].
The first indication that the S-enantiomer of a racemic \( \beta \)-agonist might cause hyperreactivity originates from experiments in guinea pigs, demonstrating that infusion of S-isoprenaline for 1 hour increased airway reactivity to histamine [479] or the peptide bombesin [480]. Salbutamol, worldwide the most commonly used \( \beta \)-agonist, is studied most extensively for possible detrimental effects of the S-enantiomer. In vitro radioligand binding assays have shown that R-salbutamol is a potent ligand for the \( \beta_2 \)-adrenoceptor, having approximately 100 times greater receptor affinity than S-salbutamol [481]. Concerns about potential adverse effects of S-salbutamol seem to be supported by a number of results obtained from animal and in vitro models. Preincubation with S-salbutamol increased reactivity to carbachol, but not histamine or ovalbumin, in tracheal smooth muscle preparations from guinea pigs sensitized to ovalbumin [482]. In addition, in human isolated bronchus, an increased response to histamine and LTC\textsubscript{4}, but not to methacholine, electric field stimulation or bradykinin, was observed after preincubation with S-salbutamol. In contrast, however, the contractile response to anti-human IgE was attenuated by S-salbutamol [483]. Some studies have demonstrated proallergic properties of S-salbutamol. Thus, S-salbutamol increased the IgE-induced production of histamine and IL-4 in murine mast cells [484], activated pro-constrictory and pro-inflammatory pathways in human bronchial smooth muscle cells [485] and additional presence of S-salbutamol eliminated the anti-inflammatory influences of R-salbutamol on antigen specific T-cell lines, including inhibition of proliferation and cytokine production [486]. Enhanced airway hyperreactivity in guinea pigs also has been demonstrated \textit{in vivo} [487] and changes in the cholinergic system may be of importance, as indicated by the observation that S-salbutamol-induced airway hyperreactivity to histamine was inhibited by vagotomy in guinea pigs [488]. Recently, it has been observed in a mouse asthma model that both R- and S-salbutamol reduced airway eosinophil trafficking and mucus hypersecretion. However, S-salbutamol increased allergen-induced airway edema and hyperresponsiveness [489].

Another approach to study the effects of the enantiomers of salbutamol in airway smooth muscle is measuring changes in the intracellular calcium concentration. It was demonstrated in isolated bovine tracheal smooth muscle cells, that S-salbutamol, but not R-salbutamol, enhanced the increase in intracellular calcium induced by carbachol [490]. Furthermore it was shown that S- and RS-salbutamol increased basal intracellular calcium levels concentration dependent in the nanomolar range, which was accompanied by shortening of the cells [491]. This increase in intracellular calcium was insensitive to the \( \beta_2 \)-adrenoceptor antagonist ICI 118,551, whereas the muscarinic receptor antagonist atropine inhibited this increase completely, suggesting involvement of a cholinergic component. This was supported by the observed binding of S-salbutamol to muscarinic receptors. Recently, in line with these results it has been reported that treatment of human bronchial smooth muscle cells for 24 hours with S-salbutamol significantly increased intracellular Ca\textsuperscript{2+}-responses of these cells upon stimulation with methacholine [485]. Over the years, clinical data have suggested that the S-enantiomer of racemic \( \beta_2 \)-adrenoceptor agonists may cause airway hyperreactivity [473] and even contribute to increased asthma death [478]. Furthermore, studies in children [492-494] and adults [495,496] with asthma or
chronic obstructive pulmonary disease (COPD) have suggested that R-salbutamol offers better efficacy and safety benefits compared with racemic salbutamol. In addition, it has been suggested that adverse effects of S-salbutamol might result from differences in pharmacokinetics. Thus, the metabolic clearance of S-salbutamol is slower than that of R-salbutamol, causing much higher plasma levels of S-salbutamol than R-salbutamol [474,497-501]. The resultant accumulation of S-salbutamol could therefore contribute to the potential adverse effects.

Epidemiological, animal, in vitro and clinical studies purporting to show deleterious effects of S-salbutamol are still in discussion and adverse effects of the S-enantiomer present in racemic β-agonist drugs are still subject of controversy. Thus, in guinea pigs it has been found that both basal airway reactivity and allergen-induced hyperreactivity towards histamine were not affected by S-salbutamol [502]. In patients with mild to moderate asthma other groups failed to find any adverse effects of S-salbutamol on bronchoprotection and bronchodilation [503,504] and several clinical studies in patients with asthma or COPD were unable to demonstrate advantage of R-salbutamol over the racemate regarding bronchodilation and airway reactivity [475,505-508]. Taken together, although some preclinical data remain intriguing, available clinical data provide little convincing support for adverse effects of S-salbutamol in asthmatic patients and the issue is at best inconclusive.
Scope of the thesis

β-Adrenergic agonists are being in use to treat asthma for more than a century. Inhalation of β-agonists has proven to be very effective for the acute relief of bronchoconstriction. In this respect, chronic use of β-agonists is not infrequent for patients with asthma [82,415,416]. However, since the increased death rates in asthma in the mid-1960s was associated with the increased use of β2-agonists, waves of concern regarding adverse effects of β-agonists have emerged.

The efficacy of these drugs in asthma mainly resides on the functional antagonism counteracting the bronchoconstrictor effects of mediators and neurotransmitters released in airway inflammation [97]. However, it is well known that during a severe exacerbation patients with asthma have a reduced bronchodilator response to β2-adrenoceptor agonists [417]. Since many mediators and neurotransmitters in allergic airway inflammation can activate PKC, this enzyme may be active in inducing heterologous β2-adrenoceptor desensitization. Indeed, in airway smooth muscle [360,509] and in peripheral blood lymphocytes [510], it has been demonstrated that Gq coupled receptor-mediated or phorbol ester-induced PKC activation may lead to heterologous β2-adrenoceptor desensitization. To investigate the functional role of contractile agonist-induced activation of PKC, we examined in chapter 2 the effects of the specific PKC-inhibitor GF 109203X on isoprenaline-induced relaxation of bovine tracheal smooth muscle contracted by various concentrations of methacholine and histamine.

In addition to an acutely reduced bronchodilator response to β-agonists during severe exacerbations, presumably as a result of heterologous β2-adrenoceptor desensitization, it has been shown that prolonged β-agant therapy can diminish the efficacy of these drugs [85,511], possibly as a consequence of homologous β2-adrenoceptor desensitization involving activated GRKs [256,297]. Heterologous and homologous desensitization, however, appear not to be completely independent processes and the interaction between the two processes may contribute additionally to the adverse effects of β-agonists. It has been reported that PKC-mediated phosphorylation upregulates the activity of GRK2 [369] and also targets this kinase to the plasma membrane [370]. Little is known, however, about the functional consequences of these interactions. To examine whether this concept is functionally operative in airway smooth muscle, we investigated in chapter 3 the capacity of PKC to regulate homologous β2-adrenoceptor desensitization in bovine tracheal smooth muscle.

In chapter 4, we explored the concept of heterologous regulation of homologous desensitization of the β2-adrenoceptor into further detail on the level of intracellular Ca2+-homeostasis, by investigating the role of PKC in attenuating the effectiveness of isoprenaline against methacholine-induced Ca2+-mobilization and Ca2+-influx in isolated bovine tracheal smooth muscle cells.

Another concern of prolonged use of β-agonists is the possibility of a paradoxical up-regulation of neural and non-neural processes that counteract β2-adrenoceptor function,
possibly by the S-enantiomer present in the racemic mixture of β2-agonists that are currently being used [477]. To date, a lot of controversial evidence has been found with regard to the putative adverse effects of S-salbutamol and possible mechanisms remain unclear. Among all diverse observations made, the finding that S-salbutamol increases the intracellular free Ca\(^{2+}\)-concentration [485,491] and enhances contractile agonist-induced Ca\(^{2+}\)-mobilization [490] in native airway smooth muscle cells, possibly by means of a cholinergic mechanism, is a fascinating observation which deserves further investigation. To this purpose, in chapter 5 we have thoroughly compared the effects of R- and S-salbutamol on methacholine and histamine-induced Ca\(^{2+}\) responses in Fura-2AM-loaded bovine tracheal smooth muscle cells, using both cell suspension spectrofluorometry and single cell fluorescence microscopy.

COMT is a very important enzyme in the inactivation of endogenous and exogenous catecholamines. Preliminary results obtained in our laboratory had shown a possible link between PKC and COMT, in that activated PKC may potentiate COMT activity. By reducing the β-adrenergic response, both PKC and COMT, as well as the possible interaction between the two, may play an important role in diminishing an important endogenous β-adrenergic defence in (severe) asthma. In chapter 6, we investigated the interactive role of PKC and COMT in reducing the β2-adrenoceptor responsiveness in bovine tracheal smooth muscle towards the catecholamine isoprenaline.

Finally, to exert their relaxing effects, β-agonists have to counteract mechanisms involved in airway smooth muscle contraction. Interestingly, airway smooth muscle contraction caused by full muscarinic agonists is relatively resistant to relaxation by β2-adrenoceptor agonists, compared to partial muscarinic agonists, possibly as a result of the considerable transduction reserve of full muscarinic agonists [120]. Indeed, it has been demonstrated that the full agonist acetylcholine induces maximal force development by occupying only 4% of the available muscarinic receptors (large receptor reserve), whereas partial agonist McN-A-343 has to occupy 80% of the receptors (low receptor reserve) to achieve the same degree of muscle shortening [512,513]. In addition, large differences between full and partial muscarinic agonists regarding Ca\(^{2+}\)-mobilizing capacity have been described [157]. Remarkably, despite these large differences, the partial agonists pilocarpine and McN-A-343 are still capable to induce approximately 85% of the maximal contraction induced by the full agonist methacholine. A putative explanation for this phenomenon is that partial muscarinic agonists are more depend on Ca\(^{2+}\)-sensitization than full muscarinic agonists, as it was recently reported that contractile stimuli do not exert their effects only by increasing the intracellular Ca\(^{2+}\)-concentration, but also by increasing Ca\(^{2+}\)-sensitivity of the smooth muscle. One of the main regulators involved in this Ca\(^{2+}\)-sensitization is Rho-kinase. To further elucidate mechanisms underlying the differences between full and partial muscarinic receptor agonists, we investigated in chapter 7 the contribution of Rho-kinase to ASM-contraction, Ca\(^{2+}\)-mobilization and Ca\(^{2+}\)-influx in response to the full muscarinic agonist methacholine and the partial muscarinic agonists pilocarpine and McN-A-343. In this context, it is very conceivable that contractile agonists that significantly rely on Ca\(^{2+}\)-
sensitization for their contraction are more easily counteracted by β-agonists, as small changes in intracellular Ca\(^{2+}\) would have large effects on airway smooth muscle tone.

**References**


Chapter 1


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Chapter 1

General introduction


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