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Ca²⁺ Dynamics and Ca²⁺ Sensitization in the Regulation of Airway Smooth Muscle Tone

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Additional information is available at the end of the chapter

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Abstract

Airway smooth muscle tone is ultimately generated by phosphorylation of myosin light chain, which is regulated by the balance between concentrations of Ca²⁺ and sensitivity to Ca²⁺ in the cytosolic side. The former is due to the Ca²⁺ influx passing through ion channels (Ca²⁺ dynamics), leading to activation of myosin light chain kinase, and the latter is due to Rho-kinase (Ca²⁺ sensitization), leading to the inactivation of myosin phosphatase. Alterations to contractility and to the proliferative phenotype, which are influenced by Ca²⁺ dynamics and Ca²⁺ sensitization, are involved in the pathophysiology of asthma and chronic obstructive pulmonary disease (COPD). Ca²⁺ dynamics are mainly due to store-operated capacitative Ca²⁺ influx and receptor-operated Ca²⁺ influx, and partly due to L-type voltage-dependent Ca²⁺ (VDC) channels. Large-conductance Ca²⁺-activated K⁺ (K_{Ca}, BK_{Ca}, Maxi-K⁺) channels are activated by G_s connected to β_2 -adrenoceptors, whereas these channels are inhibited by G_i connected to M₂ muscarinic receptors. VDC channel activity regulated by K_{Ca} channels contributes to not only functional antagonism between β_2 -adrenoceptors and muscarinic receptors but also to synergistic effects between β_2 -adrenoceptor agonists and muscarinic receptor antagonists. Moreover, an increase in Ca²⁺ influx via the K_{Ca}/VDC channel linkage causes airflow limitation and β_2 -adrenergic desensitization. In contrast, an increase in sensitivity to Ca²⁺ via Rho-kinase causes airflow limitation, airway hyperresponsiveness, β_2 -adrenergic desensitization, and airway remodeling. These airway disorders are characteristic features of asthma and COPD. K_{Ca} channels are regulated by trimeric G proteins (G_s, G_i), and Rho-kinase is regulated by a monomeric G protein (RhoA). Therefore,

Ca^{2+} dynamics due to G proteins/ K_{Ca} /VDC channel linkage and Ca^{2+} sensitization due to RhoA/Rho-kinase processes are therapeutic targets for these diseases.

Keywords: Ca^{2+} -activated K^+ channels, β_2 -adrenoceptors, G proteins, Rho-kinase, Intrinsic efficacy

1. Introduction

Contractility of airway smooth muscle is involved in airflow limitation, which is implicated in the pathophysiology of asthma and chronic obstructive pulmonary disease (COPD). Spasmogens act on trimeric G protein-coupled receptors (GPCRs), such as acetylcholine (ACh), histamine, leukotrienes, and prostaglandins, to mediate airway smooth muscle contraction. Airway smooth muscle tone is ultimately regulated by the activation of myosin light chain (MLC); MLC is phosphorylated via myosin light chain kinase (MLCK) and dephosphorylated via myosin phosphatase (MP). Activation of MLCK contracts airway smooth muscle mediated by Ca^{2+} -dependent mechanisms, which is due to increased concentrations of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) via a Ca^{2+} influx through Ca^{2+} channels (Ca^{2+} dynamics). In contrast, inactivation of MP contracts airway smooth muscle by Ca^{2+} -independent mechanisms, which are due to an increase in the sensitivity to Ca^{2+} via Rho-kinase, a protein affected by RhoA, a monomeric G protein (Ca^{2+} sensitization) [1]. RhoA/Rho-kinase processes are widely distributed in tissues including the respiratory system and regulated by agonists for GPCRs.

β_2 -adrenoceptor agonists and muscarinic receptor antagonists counteract spasmogen-induced contraction with reducing $[\text{Ca}^{2+}]_i$ (antagonizing Ca^{2+} dynamics). β_2 -adrenoceptor agonists also suppress airway smooth muscle contraction by reducing sensitivity to Ca^{2+} (antagonizing Ca^{2+} sensitization) [1, 2]. Inhibition in both Ca^{2+} dynamics and Ca^{2+} sensitization is involved in the effects of β_2 -adrenoceptor agonists against spasmogen-induced contraction. Moreover, β_2 -adrenoceptor agonists relax airway smooth muscle via 3'-5'-cyclic adenosine monophosphate (cAMP)-dependent protein kinase (protein kinase A: PKA), leading to inactivation (phosphorylation) of MLCK. Large-conductance Ca^{2+} -activated K^+ (K_{Ca} , BK_{Ca} , Maxi-K^+) channels are markedly activated by PKA-induced phosphorylation [3, 4, 5, 6] and G_s -induced action (G , a stimulatory trimeric G protein of adenylyl cyclase) [4, 5, 6, 7]. K_{Ca} channels are activated by β_2 -adrenoceptor agonists via G_s and suppressed by muscarinic receptor agonists via G_i , an inhibitory trimeric G protein of adenylyl cyclase [7, 8]. Since K_{Ca} channels have a large conductance of outward currents and exist innumerable on the cell membrane in airway smooth muscle [9], the opening of these channels also regulates airway smooth muscle tone mediated by membrane potential-dependent Ca^{2+} influx (Ca^{2+} dynamics), such as L-type voltage-dependent Ca^{2+} (VDC) channels [10]. Therefore, not only Ca^{2+} signaling (Ca^{2+} dynamics and Ca^{2+} sensitization) but also K_{Ca} channels play a key role in the functional antagonism between β_2 -adrenoceptors and muscarinic receptors in airway smooth muscle.

Alterations of contractile phenotype, i.e. hyperresponsiveness to contractile agents (airway hyperresponsiveness) or hyporesponsiveness to relaxant agents (β_2 -adrenergic desensitiza-

tion), occurs due to intrinsic or extrinsic factors involved in the pathophysiology of asthma. Dysfunctional contractility, which is a characteristic feature of patients with asthma, may depend on Ca²⁺ signaling (Ca²⁺ dynamics and Ca²⁺ sensitization) and K_{Ca} channels [1, 6, 11, 12]. Furthermore, airway smooth muscle cells have the ability to change the degree of various functions, such as contractility, proliferation, migration, and synthesis of inflammatory mediators [1, 13, 14]. The plasticity from a contractile phenotype to other phenotypes (proliferation, migration, or secretion of chemical mediators) may enhance airway inflammation, leading to airway remodeling, which is also characterized in asthma. This phenotype change may also be associated with Ca²⁺ signaling (Ca²⁺ dynamics and Ca²⁺ sensitization) and K_{Ca} channels.

Ca²⁺ signaling and K_{Ca} channels involved in the regulation of airway smooth muscle tone may be therapeutic targets in asthma and COPD [1, 6, 10, 11, 12, 15]. To elucidate the cause of the pathophysiology in asthma and COPD, and to establish a rational bronchodilator use for these diseases, the mechanisms underlying the regulation of airway smooth muscle tone via β_2 -adrenergic and muscarinic receptors were examined by using physiological techniques such as single-channel recording in tracheal smooth muscle cells, isometric tension recordings of isolated tracheal smooth muscle and simultaneous recording of isometric tension and F₃₄₀/F₃₈₀ in Fura-2-loaded tracheal smooth muscle. In this chapter, the functional characteristics of airway smooth muscle involved in alterations of contractile and proliferative ability are focused on Ca²⁺ signaling (Ca²⁺ dynamics and Ca²⁺ sensitization) mediated by G protein/K_{Ca}/VDC linkage and RhoA/Rho-kinase processes.

2. Mechanism of airway smooth muscle tone

Contractile agonists acting on GPCRs cause contraction of airway smooth muscle with increasing [Ca²⁺]_i mediated by Ca²⁺ influx passing through Ca²⁺ channels (Ca²⁺ dynamics). When ligands are connected to the GPCRs, receptor-operated Ca²⁺ (ROC) influx is activated [16], and Ca²⁺ is released from sarcoplasmic reticulum (SR) via the production of inositol-1,4,5-triphosphate (IP₃). This Ca²⁺ release activates store-operated capacitative Ca²⁺ (SOC) influx (Figure 1) [17]. Moreover, VDC channels are mainly activated by membrane depolarization under the condition of high K⁺ at the extracellular side. Ca²⁺ influx passing through VDC channels contributes to high K⁺-induced contraction. In contrast, VDC is partly involved in the GPCR-mediated Ca²⁺ influx [10]. An increase in [Ca²⁺]_i enhances the binding of Ca²⁺ to calmodulin (CaM), a calcium-binding messenger protein. MLCK activity is augmented by a Ca²⁺-CaM complex (Ca²⁺/CaM), and MLC is phosphorylated (activated) by MLCK [18], leading to contraction of airway smooth muscle (Ca²⁺-dependent contraction: Ca²⁺ dynamics) [10, 17, 19]. After activated MLC is dephosphorylated (inactivated) by MP, contraction is reversed to relaxation (Figure 1). On the other hand, contractile agonists activate RhoA mediated by stimulating GPCRs. RhoA is activated by binding to GTP (RhoA-GTP: active form of RhoA). Rho-kinase is activated by RhoA-GTP, and MP is phosphorylated by Rho-kinase (MP inactivation) (Figure 1) [20, 21]. MP is also phosphorylated by CPI-17, which is another potential

mediator regulated by protein kinase C [22]. Since MLC activity is sustained, not suppressed, by loss of MLC dephosphorylation via inactivation of MP, airway smooth muscle tone is enhanced without increasing $[Ca^{2+}]_i$ (Ca^{2+} -independent contraction: Ca^{2+} sensitization) [19, 23]. Airway smooth muscle tone is regulated by the degree of MLC phosphorylation mediated by both MLCK and MP activity. Alterations of contractile phenotype, which are due to both Ca^{2+} dynamics and Ca^{2+} sensitization, have clinical relevance to airflow limitation, airway hyperresponsiveness, and reduced responsiveness to β_2 -adrenoceptor agonists (β_2 -adrenergic desensitization), which are implicated with the pathophysiology of obstructive pulmonary diseases, such as asthma and COPD [1].

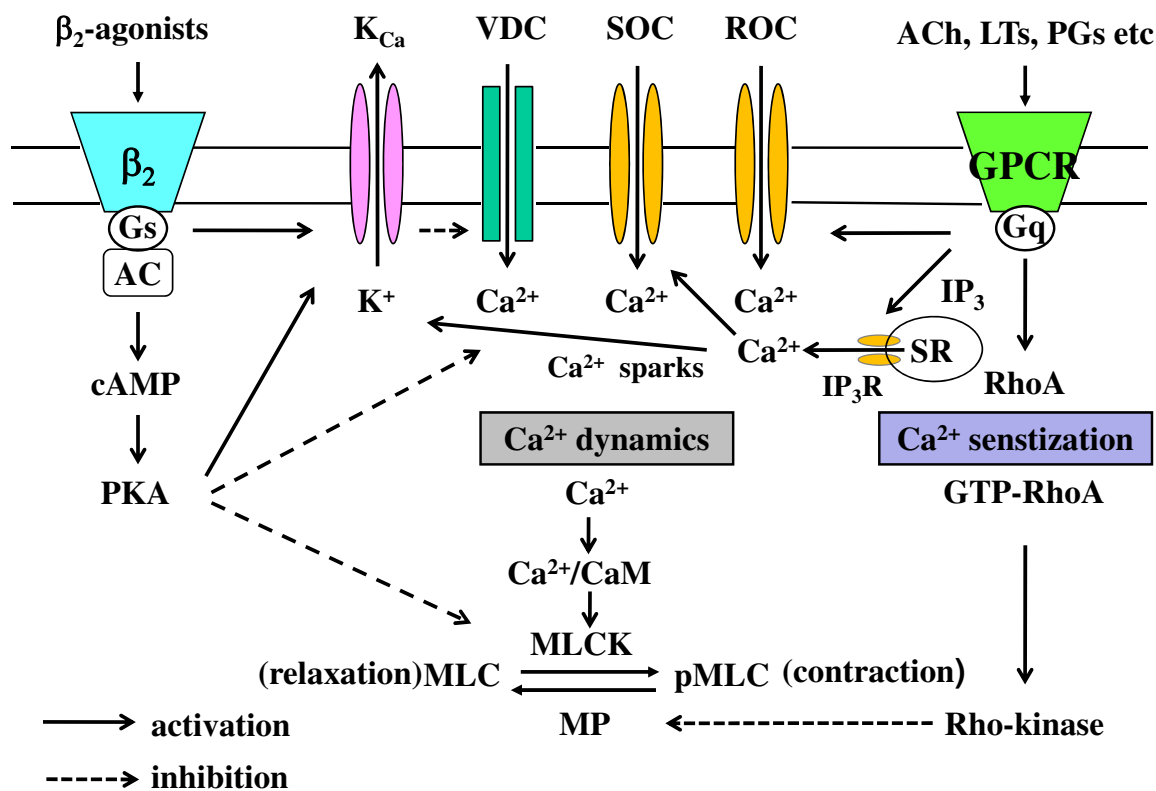


Figure 1. Role of Ca^{2+} dynamics and Ca^{2+} sensitization in the regulation of airway smooth muscle tone. Ca^{2+} signaling via Ca^{2+} dynamics and Ca^{2+} sensitization contributes to the functional antagonism between β_2 -adrenoceptor agonists and contractile agonists (such as histamine, ACh, LTs, and PGs), acting on GPCRs. MLC phosphorylation (pMLC), which is regulated by a balance between MLCK and MP, is fundamental for controlling contraction in airway smooth muscle. GPCR-related agents cause Ca^{2+} influx by activating ROC and cause Ca^{2+} release from SR by producing IP₃. The latter process induces Ca^{2+} influx via activating SOC. An increase in intracellular concentrations of Ca^{2+} mediated by these processes enhances the binding of Ca^{2+} to CaM. A Ca^{2+} -CaM complex (Ca^{2+} /CaM) augments MLCK activity, leading to MLC phosphorylation (Ca^{2+} dynamics: Ca^{2+} -dependent mechanisms). On the other hand, contractile agonists activate RhoA by acting on G-protein-coupled receptors. Rho-kinase activated by GTP-RhoA phosphorylates (inactivates) MP, leading to MLC phosphorylation (Ca^{2+} sensitization: Ca^{2+} -independent mechanisms). ACh: acetylcholine, LTs: leukotrienes, PGs: prostaglandins, β_2 : β_2 -adrenoceptors, GPCRs: G-protein-coupled receptors, AC: adenylyl cyclase, ROC: receptor-operated Ca^{2+} influx, SOC: store-operated Ca^{2+} influx, IP₃: inositol-1,4,5-triphosphate, SR: sarcoplasmic reticulum, PKA: protein kinase A, CaM: calmodulin, MLCK: myosin light chain kinase, MLC: myosin light chain, MP: myosin phosphatase, K_{Ca} : large-conductance Ca^{2+} -activated K^+ channels, VDC: L-type voltage-dependent Ca^{2+} channels Illustrated based on ref. [1]

3. Airway smooth muscle tone regulated by K_{Ca} channels

3.1. Characteristics and physiological roles of K_{Ca} channels

3.1.1. Structure of K_{Ca} channels

K_{Ca} channels are composed of a tetramer formed by pore-forming α -subunits along with accessory β -subunits, and these channels are activated by increased membrane potential and increased [Ca²⁺]_i. The α -subunit is ubiquitously expressed by mammalian tissues and encoded by a single gene (Slo, KCNMA1) [24, 25]. The α -subunit transmembrane domains comprise seven membrane-spanning segments (S0-S6) with extracellular loops and share homology with all voltage-gated K⁺ channels with six transmembrane domains (S1-S6) and a pore helix. S1-S4 are arranged in a bundle that forms the voltage-sensing component, and S5-S6 and pore helices contribute to form the pore-forming component and the K⁺ selective filter [26]. The C-terminal tail confers the Ca²⁺-sensing ability of the K_{Ca} channels, involving a pair of Ca²⁺-sensing domains that regulate the conductance of K⁺ (RCK), i.e., RCK1 and RCK2 [27]. Although the Ca²⁺ sensor of the K_{Ca} channels has high specificity for Ca²⁺, other factors including divalent cations also influence the opening of these channels. Magnesium (Mg²⁺) enhances activation of these channels via a distinct binding site in the voltage sensor and RCK1 domain [28]. On the other hand, intracellular protons (H⁺) attenuate the opening of the K_{Ca} channels [9, 29]. K_{Ca} channels associate with modulatory β -subunits, which are expressed in a cell-specific manner and have unique regulatory actions on these channels. The β -subunits bring about diversity of the K_{Ca} channels. There are four distinct β -subunits, β 1-4, which are encoded by KCNMB1, KCNMB2, KCNMB3, and KCNMB4. These β -subunits in the K_{Ca} channels consist of two transmembrane domains with intracellular N- and C-termini and a long extracellular loop. The β 1 subunit was the first β -subunit to be cloned and is primarily expressed in smooth muscle [30].

3.1.2. Electrical characteristics of K_{Ca} channels

K_{Ca} channels are densely distributed on the cell membrane in airway smooth muscle cells and have a large conductance (about 250 pS in a symmetrical 135-150 mM K⁺ medium) [31, 32, 33], as compared to other K⁺ channels. In freshly isolated human bronchial smooth muscle cells, single currents of the K_{Ca} channels were also recorded in cell-attached patches, inside-out patches, and outside-out patches [34, 35]. These channels have a conductance of about 210 pS in symmetrical 140 mM K⁺ medium. K_{Ca} channels are highly selective for K⁺ despite their large conductance [36]. Ca²⁺ sensitivity may be increased by intracellular Mg²⁺, as is the case in vascular muscle [37]. Effects of intracellular pH (pH_i) on K_{Ca} channels have been studied in rabbit tracheal muscle by using inside-out patches [9]. K_{Ca} channel activity was markedly inhibited by intracellular acidification, by reducing the sensitivity to Ca²⁺ and also by shortening the open state of the channel. On the other hand, intracellular alkalization had an opposite effect (increasing Ca²⁺ sensitivity and lengthening the open state of the channel). Single-channel currents of K_{Ca} channels in guinea pig and canine tracheal muscle, studied in

outside-out patches, were reversibly blocked by external application of charybdotoxin (ChTX) or iberiotoxin (IbTX), selective antagonists of K_{Ca} channels. This effect was not a result of reduced current amplitude; rather, it was caused by reducing the open-state probability (nPo), the fraction of the time during which the channel is open [7, 38]. In bovine trachealis, externally applied tetraethylammonium (TEA, 1 mM) strongly reduced the amplitude of single K_{Ca} channel current, different from the effects of ChTX (100 nM) on these channels without affecting current amplitude [32]. The effect of ChTX was also reversible. In contrast, the K_{Ca} channels were not affected by 4-aminopyridine (4-AP, 1 mM) applied internally or (2 mM) externally.

3.1.3. Physiological role of K_{Ca} channels

Typical action potentials have not been found in airway muscle under physiological conditions. This lack of action potentials is believed to be due to a marked increase in K^+ conductance of the plasma membrane upon depolarization [39]. Thus, when the K^+ conductance of the membrane is reduced by blocking K^+ channels, one would expect an increase in excitability. In airway smooth muscle that is only weakly excitable, spontaneous phasic contractions can be initiated along with electrical activities by applying K^+ channel blocking agents, such as TEA, 4-AP, ChTX and IbTX [40]. Some of these contractions are accompanied by electrical activity. These observations suggest that outward K^+ currents passing through K_{Ca} channels may be functioning in an important regulatory role in these smooth muscle cells [41].

In excitation-contraction coupling of smooth muscle cells, local increases in Ca^{2+} concentrations occur due to focal releases of Ca^{2+} through ryanodine receptors (RyR) from the sarcoplasmic reticulum (SR), termed Ca^{2+} sparks [42]. Hundreds of K_{Ca} channels are opened by the Ca^{2+} sparks from SR close to the sarcolemma, leading to spontaneous outward currents (STOCs) (Figure 1). The coupling of ryanodine-mediated Ca^{2+} sparks to K_{Ca} channel-mediated STOCs is enhanced by the β_1 subunit, resulting in hyperpolarization of smooth muscle cells and the subsequent reduction of Ca^{2+} influx and initiation of muscle relaxation. In K_{Ca} channel β_1 subunit knockout mice, tracheal contraction induced by carbachol (CCh), a muscarinic receptor agonist, was enhanced as compared to wild-type mice, and not only the single channel activity of K_{Ca} channels in an inside-out patch but also STOCs in a whole cell configuration were markedly attenuated in tracheal smooth muscle cells of knockout mice as compared to wild-type mice [43]. IbTX (30 nM) enhances contraction induced by methacholine (MCh), a muscarinic receptor agonist, and verapamil, an inhibitor of VDC, suppresses the effect of IbTX on tension, demonstrating that K_{Ca} channel inhibition augments contraction via a Ca^{2+} influx through VDC channels [10].

3.2. Stimulatory regulation of K_{Ca} channels by β_2 -adrenergic receptor agonists

3.2.1. cAMP-dependent phosphorylation

The involvement of cAMP-dependent processes in K_{Ca} channel regulation has been examined in rabbit tracheal smooth muscle cells by using single-channel recording. In the presence of cAMP and adenosine triphosphate (ATP, 0.3 mM), application of PKA (10 units/ml) to the

cytosolic side of inside-out membrane patches reversibly increased the nPo of K_{Ca} channels without changes in the amplitude of single-channel currents, and the recovery from this activation was significantly delayed by okadaic acid, an inhibitor of protein phosphatases [3]. A similar effect was observed with the catalytic subunit of PKA (10 units/ml), indicating that phosphorylation of a K_{Ca} channel protein enhances the open state of the channel [3, 4]. External application of isoprenaline (0.2 μM), a β₂-adrenoceptor agonist, and okadaic acid (10 μM) also increased the activation of K_{Ca} channels in the cell-attached patch-clamp configuration, and the recovery from this activation was also significantly delayed by okadaic acid (Figure 2A) [3]. In *Xenopus* oocytes, similar results were observed in β-adrenergic action [44]. Moreover, external application of forskolin (10 μM), a direct activator of adenylyl cyclase, increased the K_{Ca} channel activity in tracheal smooth muscle cells [45]. These results are in accordance with results obtained in cultured smooth muscle cells of rat aorta using isoprenaline (10 μM), forskolin (10 μM), and dibutyryl cAMP (100 μM) in cell-attached patches and by using PKA (0.5 μM) and cAMP (1 μM) in inside-out patches [46]. These findings demonstrate that β₂-adrenoceptor agonists augment K_{Ca} channel activity via PKA-mediated phosphorylation in airway smooth muscle.

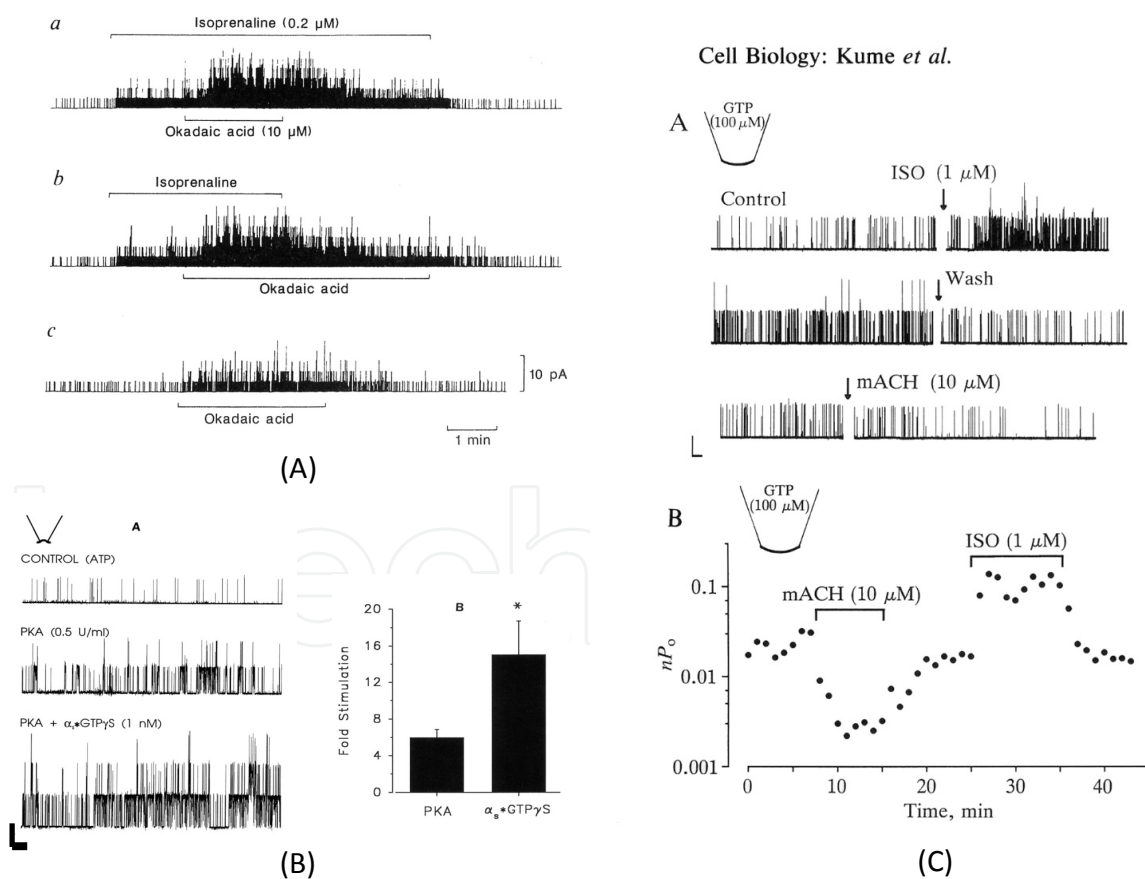


Figure 2. Stimulation and inhibition of K_{Ca} channels by β₂-adrenoceptor and muscarinic receptor agonists in single-channel recording of tracheal smooth muscle cells. A: A continuous recording of the effects of external application of isoprenaline (0.2 μM) and okadaic acid (10 μM) on K_{Ca} channels in a cell-attached configuration held at -40 mV. Isoprenaline increased K_{Ca} channel activity, and okadaic acid enhanced the effects of isoprenaline on these channels (up-

per trace). The time course for washing out the effects of isoprenaline was markedly prolonged in the presence of okadaic acid (middle trace). These results demonstrate that K_{Ca} channel activity is regulated by phosphorylation via PKA. Okadaic acid augmented K_{Ca} channel activity, demonstrating that phosphatase activity is still intact in this experimental condition (lower trace). B: A continuous recording of the effects of PKA and α_s^* GTP γ S on K_{Ca} channels in an inside-out patch held at 0 mV (left panel). PKA (0.5 U/ml) maximally increased K_{Ca} channel activity, and addition of the α_s^* GTP γ S (1 nM) enhanced K_{Ca} channel activity prestimulated by PKA (0.5 U/ml), indicating that α_s activates K_{Ca} channels independent of PKA. Calibration bars, 3 pA and 4 s. Fold stimulation of channel activity are shown under the condition of addition of PKA (0.5 U/ml) and subsequently by addition of α_s^* GTP γ S (1 nM) (right panel). C: A continuous recording of the effects of ISO (1 μ M) and mACh (10 μ M) on K_{Ca} channels in an outside-out patch held at 0 mV. External application of ISO increased K_{Ca} channel activity, whereas, following washout, mACh decreased this channel activity (upper trace), indicating that K_{Ca} channels are key molecules for the functional antagonisms between these two receptors. Calibration bars, 3 pA and 10 s. Relationship between nPo and time for an experiment similar to the upper trace with agonists added in reverse order (lower trace). PKA: protein kinase A, α_s : α -subunit of G_s , which is stimulatory G protein of adenylyl cyclase, ISO: isoprenaline, mACh: methacholine, K_{Ca} : large-conductance Ca^{2+} -activated K^+ channels, nPo: open-state probability, U: unit. Cited from ref. [3, 4, 7].

3.2.2. Membrane-delimited activation by G_s 30 nM

Activation of K_{Ca} channels by isoprenaline is mediated by the α -subunit (α_s) of the stimulatory G protein of adenylyl cyclase (G_s), independent of cAMP-dependent protein phosphorylation [4, 7]. In porcine, canine and ferret tracheal muscle cells, isoprenaline increased the activation of K_{Ca} channels in outside-out patches when guanosine triphosphate (GTP, 100 μ M) was present at the cytosolic side of the patch. A similar increase in K_{Ca} channel activity was also observed even when phosphorylation was inhibited by the nonmetabolizable ATP analog, adenosine 5'-[β , γ -imido] diphosphate (ATP [β ; γ NH], AMP-PNP (1 mM)) [4, 7]. In inside-out patch configuration with a patch pipette containing isoprenaline (1 μ M), nonhydrolyzable GTP analog guanosine 5'-O-(3-thiotriphosphate) (GTP- γ -S, 100 μ M) similarly potentiated the K_{Ca} channel activity. The recombinant α_s proteins preincubated with GTP- γ -S (α_s^* GTP γ S, 100-1000 pM) increased the channel activity in a concentration-dependent manner when applied to the cytosolic side of inside-out patches [7]. The maximum effects of α_s^* GTP γ S were observed at 1000 pM, and the nPo of K_{Ca} channels was augmented to approximately 16-fold. On the other hand, α_s preincubated with guanosine 5'-O-(2-thio-diphosphate) (GDP- β -S) had no effect on these channels. These results indicate that the K_{Ca} channels are directly activated by α_s (membrane-delimited action) and that cAMP-dependent phosphorylation is not required. A direct action of G_s protein on the K_{Ca} channels has also been demonstrated in channels from rat or pig myometrium incorporated into planar lipid bilayers, by using GTP- γ -S and AMP-PNP [47]. β_2 -adrenoceptor agonists act on smooth muscle without the intracellular signal transduction processes (the cAMP-PKA pathway).

3.2.3. Dual regulation by cAMP-dependent and -independent processes

To examine whether receptor-channel coupling could occur in β_2 -adrenergic action on K_{Ca} channels, isoprenaline was applied to outside-out patches in the presence of GTP (100 μ M) and AMP-PNP (1 mM), the competitive ATP inhibitor, in porcine tracheal smooth muscle cells (Figure 3) [4]. Isoprenaline (1 μ M) markedly activated K_{Ca} channel activity without an alteration in current amplitude and returned to the control level within 5 min after drug washout. The nPo of the channels was increased to approximately fivefold in the presence of isoprenaline. This result was roughly equivalent to the level of channel stimulation previously

reported in outside-out experiments in the absence of ATP, but without AMP-PNP. Consistent with a membrane-delimited, G protein-dependent coupling mechanism, addition of guanine nucleotides to the cytosolic side stimulated K_{Ca} channel activity in inside-out patches exposed to isoprenaline on the external side. Internal application of GTP (100 μM) also led to a marked increase in K_{Ca} channel activity; the nPo of the channels was increased to an approximately equivalent fold, as compared to the experimental condition when isoprenaline was applied to the outside-out patches in the presence of GTP. Stimulation of channel activity resulted in an apparent shift in the relationship between voltage and nPo by 10-15 mV after the addition of 100 μM GTP.

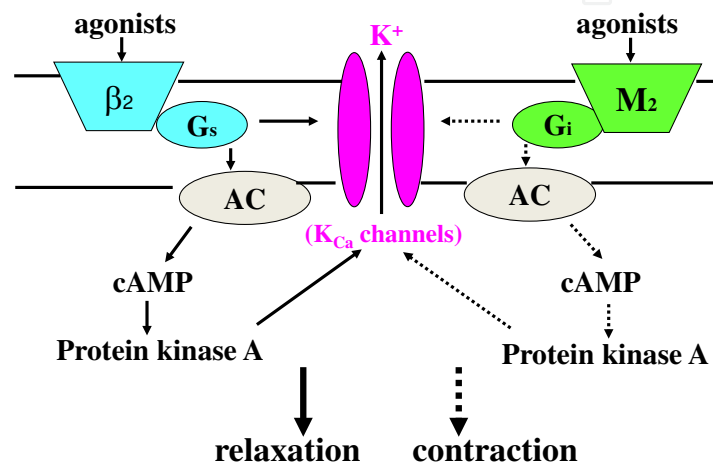


Figure 3. Dual pathway and dual regulation of K_{Ca} channels in the functional antagonisms between β_2 -adrenoceptors and muscarinic receptors. At least two mechanisms are involved in activation of K_{Ca} channels following the β_2 -adrenoceptor activation; one is mediated through cAMP-dependent channel phosphorylation and the other through direct, cAMP-independent regulation by G_s protein (dual pathway). In contrast, in the muscarinic suppression of K_{Ca} channels, G_i proteins connected to M₂ receptors are involved (dual regulation). The relationship between G proteins and K_{Ca} channels, i.e. the G_s/K_{Ca} stimulatory linkage and the G_i/K_{Ca} inhibitory linkage, may play a key role in the functional antagonisms (relaxation, contraction) between β_2 -adrenoceptors and muscarinic receptors in airway smooth muscle. β_2 : β_2 -adrenoceptors, M₂: M₂ muscarinic receptors, AC: adenylyl cyclase, G_i: inhibitory G protein of adenylyl cyclase, G_s: stimulatory G protein of adenylyl cyclase, PKA: protein kinase A, K_{Ca}: large-conductance Ca²⁺-activated K⁺ channels. Illustrated based on ref. [3, 4, 7, 53].

Stimulation of K_{Ca} channels by the catalytic subunit of cAMP-dependent PKA was examined in inside-out patches. K_{Ca} channel activity was progressively stimulated by a cumulative dose-response protocol (PKA between 0.0005 and 5.0 units/ml). The maximum level of K_{Ca} channel stimulation by PKA was observed at either 0.5 or 5.0 units/ml (approximately sevenfold stimulation). At peak effect, the mean stimulation was approximately 7-fold, which was substantially less than the approximately 16-fold stimulation previously observed for 1 nM α_s *GTP γ S. To examine the dual pathway of β_2 -adrenoceptor/channel coupling, inside-out patches were stimulated to near maximum with PKA (0.5 unit/ml). This concentration was chosen since it provided near maximal stimulation in all patches and a stable stimulation of channel activity over time. Following incubation with PKA for 5 min, α_s *GTP γ S (1 nM) was added. K_{Ca} channels were potently activated by the addition of recombinant α_s protein after

stimulation by the near maximally effective concentration of PKA (Figure 2B) [4]. PKA produced an approximately 6-fold stimulation, and addition of α_s produced an approximately 15-fold increase over baseline channel activity (Figure 2B). The fold stimulation produced during the condition of combined PKA and α_s application was more than twice as great as the maximal fold stimulation that could be produced by PKA alone, suggesting that PKA and α_s affect the channels independently.

The gating kinetics of K_{Ca} channels were quantitatively examined by monitoring the effects of stimulation of channel activity by isoprenaline (outside-out patches) and by PKA and α_s (inside-out patches) at the level of channel open-time kinetics [4]. K_{Ca} channel open-times were well fit by the sum of two exponentials of mean duration τ_1 and τ_2 , similar to previous reports [8, 9, 48]. The effect of α_s on open-time kinetics was remarkably similar to that produced by isoprenaline on open-time kinetics; that is, α_s did not alter the mean lifetimes, but increased the proportion of long open-time events. In contrast, the major kinetic effect of PKA was on open-state time constants, resulting in an increase in the mean duration of the long openings. The effect of PKA on channel kinetics was distinct from that of α_s , consistent with distinct or independent modulatory effects at the channel protein.

3.2.4. Role in relaxation by β_2 -adrenergic receptor agonists

Airway smooth muscle relaxation produced by β -adrenoceptor activation is generally accompanied by membrane hyperpolarization, observed with intracellular microelectrodes in guinea pig, dog, and human tracheal muscles [49, 50], for which activation of K_{Ca} channels is thought to be responsible for the relaxation, as described earlier. This idea is supported by the observations in guinea pig and human trachealis that the relaxation by noradrenaline (1 μ M) against CCh-induced contraction was nearly blocked by ChTX (50 nM) and that the concentration-relaxation curves to β_2 -adrenoceptor agonists, such as isoprenaline and salbutamol, were selectively shifted to the right by ChTX [51, 52]. The relaxant effect of forskolin on MCh-induced contraction was also attenuated in the presence of ChTX, similar to isoprenaline [45]. Therefore, an increase in K_{Ca} channel activity may contribute to airway smooth muscle relaxation induced by β_2 -adrenoceptor agonists and cAMP-related agents. After G_s activity was irreversibly augmented by incubation with cholera toxin (2 μ g/ml) for 6 h in guinea pig trachea, MCh-induced contraction was significantly attenuated, and this effect by G_s was reversed in the presence of ChTX (100 nM) [53]. The G_s/K_{Ca} stimulatory linkage may also be involved in β -adrenergic relaxation in airway smooth muscle.

3.3. Inhibitory regulation of K_{Ca} channels by muscarinic receptor agonists

3.3.1. Membrane-delimited inhibition by G_i

When MCh (50 μ M) was applied to outside-out patches of porcine or canine tracheal muscle cells, the nPo of the K_{Ca} channel was markedly decreased without changes in the amplitude of single-channel currents [8, 54]. The decreased nPo is due to a reduction in channel open times,

probably reflecting a decrease in the Ca²⁺ sensitivity of the channel. The muscarinic inhibition of K_{Ca} channels, similar to that found in airway smooth muscle, has been reported for the circular muscle of canine colon. The inhibition of K_{Ca} channels through muscarinic activation in guinea pig and swine tracheal muscle cells may be partly responsible for the prolonged suppression by ACh of STOCs following a transient increase [55, 56]. This suppression has been observed in longitudinal muscle cells of the rabbit jejunum. As discussed by Saunders and Farley, this inhibition is difficult to explain by the depletion of intracellular Ca²⁺ stores, because it occurs even with elevated Ca²⁺ concentrations. In the porcine and canine trachealis, the inhibition of K_{Ca} channels produced by muscarinic stimulation was potentiated by cytosolic application of GTP (100 μM), and strong, irreversible, potentiation was obtained with GTP-γ-S (100 μM) [8]. On the other hand, when GDP-β-S (1 mM) was applied to the cytosolic side, muscarinic inhibition was not observed. Incubation (4-6 h) of airway smooth muscle cells with pertussis toxin (0.1-1.0 μg/ml), which blocks signal transduction through ADP ribosylation of G_i, the inhibitory G protein of adenylyl cyclase, abolished the channel inhibition by MCh, without reducing channel activity in the control state [8]. The G_i/K_{Ca} inhibitory linkage may be involved in the muscarinic action in airway smooth muscle.

3.3.2. Dual regulation by G_s and G_i

As described earlier, K_{Ca} channels are markedly activated by β₂-adrenoceptor agonists; in contrast, K_{Ca} channels are markedly suppressed by muscarinic receptor agonists via G proteins (Figure 3). The activation process is mediated by the stimulatory G protein, G_s; in contrast, the suppression process is mediated by the inhibitory G protein, G_i (dual regulation). To demonstrate the functional antagonistic, hormone-linked stimulatory and inhibitory regulation of K_{Ca} channels by G proteins at the single-channel level, isoprenaline and MCh were sequentially applied to identical outside-out patches under the condition of physiologic Ca²⁺ concentration and GTP (100 μM) [7]. External application of isoprenaline (1 μM) markedly increased K_{Ca} channel activity, and following drug washout this channel activity reversed to baseline; then, external application of MCh (10 μM) markedly decreased this channel activity (Figure 2C). Receptor-linked stimulatory and inhibitory modulation of K_{Ca} channels was not sequentially dependent as shown by an experiment in which this channel activity was inhibited by MCh and then activated by isoprenaline. Consistent with these outside-out experiments, internal addition of guanine nucleotides stimulated K_{Ca} channels when isoprenaline was present at the extracellular side in inside-out patches, and conversely, guanine nucleotides suppressed the channel activity when MCh was present at the extracellular side in inside-out patches [7]. These results indicate that the functional antagonism between β₂-adrenergic and muscarinic action converges on a single K_{Ca} channel current. Therefore, K_{Ca} channel activity plays a key role in the regulation of airway smooth muscle tone.

3.3.3. Role in contraction by muscarinic receptor agonists

After incubation of tracheal smooth muscle with pertussis toxin (1.0 μg/ml for 6 h), MCh-induced contraction was significantly attenuated, and this effect by pertussis toxin was

reversed in the presence of ChTX [53]. The G_i/K_{Ca} inhibitory linkage may be involved in the muscarinic-induced contraction in airway smooth muscle. From a functional point of view, it would be favorable to reduce the K^+ conductance of the plasma membrane to produce excitation by agonists such as ACh. G_i protein couples with the M_2 subtype of muscarinic receptors, leading to an inhibition in cAMP. These M_2 receptors exist on the surface of airway smooth muscle cells. A selective M_2 receptor antagonist (AF-DX 116, a benzodiazepine derivative) suppressed MCh-induced contraction in a concentration-dependent manner and potentiated relaxation induced by isoprenaline and forskolin in MCh-precontracted tracheal muscle [53]. AF-DX116 had no effect on isoprenaline-induced relaxation when the preparation was precontracted with histamine. The functional antagonism between isoprenaline (or forskolin) and M_2 receptor stimulation may not only be simply mediated by inhibition of adenylyl cyclase through the M_2 receptors but also be exerted by the direct inhibition of K_{Ca} channels by pertussis toxin-sensitive G_i protein through activation of muscarinic receptors, since there is evidence that the activation of K_{Ca} channels is involved in the relaxation induced by forskolin and isoprenaline. Furthermore, M_2 receptors inhibited the activity of K_{Ca} channels via dual pathways of a direct membrane-delimited interaction of $G\beta\gamma$ and activation of phospholipase C/protein kinase C [57]. In K_{Ca} channel $\beta 1$ subunit knockout mice, CCh-induced contraction and membrane depolarization in tracheal smooth muscle were enhanced as compared to wild-type mice, and these augmented effects of CCh were inhibited in the presence of AF-DX116 [43, 58]. These results indicate that the K_{Ca} channel $\beta 1$ subunit plays a functional role in opposing M_2 muscarinic receptor signaling.

3.3.4. Regulation of K_{Ca} channels by other factors (cGMP, protein kinase C)

3.3.4.1. NO, cGMP

Nitric oxide (NO), which is primarily generated by nitric oxide synthase (NOS) in the endothelium, causes relaxation of vascular smooth muscle cells via hyperpolarization of the cell membrane [59, 60]. NO also augmented the K_{Ca} channel activity in vascular smooth muscles, and NO-induced vasodilation was attenuated by blockade of the K_{Ca} channel activity [61]. The NO/3'-5'-cyclic guanosine monophosphate (cGMP) pathway plays an important role in relaxation of smooth muscle including vessels and airways. K_{Ca} channels were markedly enhanced by cGMP-mediated processes, suggesting that activation of these channels leads to cGMP-induced relaxation of smooth muscle [62, 63]. The K_{Ca} channel α -subunit null mice had increased vascular smooth contraction as compared to wild-type mice [64]. This phenomenon was due to an impaired response to cGMP-dependent vasorelaxation, indicating that the K_{Ca} channel is an important effector for cGMP-mediated action. Protein kinase G (PKG) was involved in this activation of K_{Ca} channels via the NO/cGMP pathway [65, 66]. Activation of K_{Ca} channels via dopamine receptors occurs through PKG and mediates relaxation in coronary and renal arteries [67]. PKG may be cross-activated by cAMP to stimulate K_{Ca} channels [68]. Moreover, dual pathways of K_{Ca} channel modulation by NO have been demonstrated; these pathways are the PKG-dependent pathway [69] and the direct activation of NO with the

channel protein [70]. Since the stimulatory effect of NO on K_{Ca} channels was abolished by knockdown of the β -subunit with antisense, the β -subunit acts as a mediator of NO [71].

3.3.4.2. Protein kinase C

K_{Ca} channels are activated via phosphorylation of their channels by PKA and PKG, as described earlier. However, the effects of protein kinase C (PKC) on these channels are still controversial. PKC enhanced the activity of K_{Ca} channels in rat pulmonary arterial smooth muscle [72]. In contrast, PKC reversed cAMP-induced activation of these channels [73]. The phosphorylation by PKC acts on K_{Ca} channels via direct inhibition and also acts as a switch to influence the effects of PKA and PKG [74, 75]. In addition to these pathways, c-Src and tyrosine kinase suppressed the activity of the K_{Ca} channels in coronary and aortic myocytes [76], whereas cSrc-induced phosphorylation augmented these channels in HEK 293 cells [77].

3.3.4.3. Redox and ROS

Reactive oxygen species (ROS) synthesized in endothelial and smooth muscle cells exert physiological and pathophysiological effects on smooth muscle via altering intracellular reduction and/or oxygen (redox) status [78]. The redox state influences the gating of K_{Ca} channels [79]. However, the effects of redox are complex. Preferential oxidation of methionine increased the activity of K_{Ca} channels, whereas oxidation of cysteines reduced the channel activity [80, 81]. K_{Ca} channel activity was enhanced by hydrogen peroxide (H₂O₂) in pulmonary arterial smooth muscle, resulting in vasodilation mediated by membrane hyperpolarization [82]. Hydrogen peroxide (H₂O₂) may directly bind to K_{Ca} channels to regulate them, or it may activate these channels via the phospholipase A₂-arachidonic acid pathway and metabolites of lipoxygenase [83]. On the other hand, H₂O₂ caused contraction of tracheal smooth muscle in a concentration-dependent fashion and elevation of [Ca²⁺]_i [84]. Moreover, peroxynitrite (OONO⁻), an oxidant generated by the reaction of NO and superoxide, caused contraction of the cerebral artery by inhibiting K_{Ca} channel activity [85].

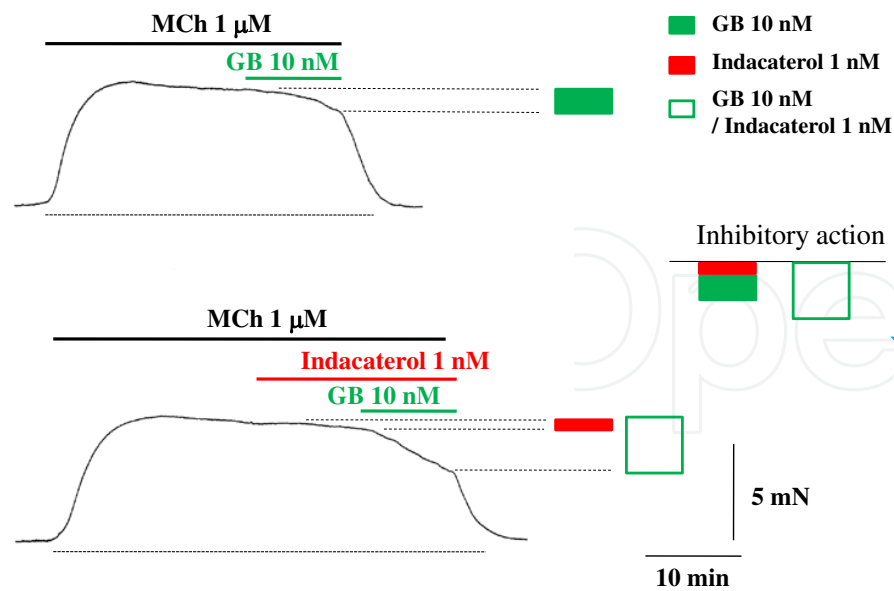
3.3.4.4. Arachidonic acid

Arachidonic acid and its metabolites such as 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) play an important role in the regulation of vascular smooth muscle tone. Arachidonic acid and EETs caused vasodilation mediated by increasing K_{Ca} channel activity [86, 87]. In airway smooth muscle, 20-HETE also caused relaxation with membrane hyperpolarization via activation of K_{Ca} channels [88]. On the other hand, 20-HETE is a vasoconstrictor. K_{Ca} channel activity was inhibited by 20-HETE, and this phenomenon is mediated by PKC [89]. The vasoconstriction induced by 20-HETE was also attenuated by increasing K_{Ca} channel activity [89]. Acute hypoxia reduced the generation of 20-HETE, and subsequently the inhibitory action of 20-HETE on K_{Ca} channels was removed in cerebral arterial smooth muscle cells [90].

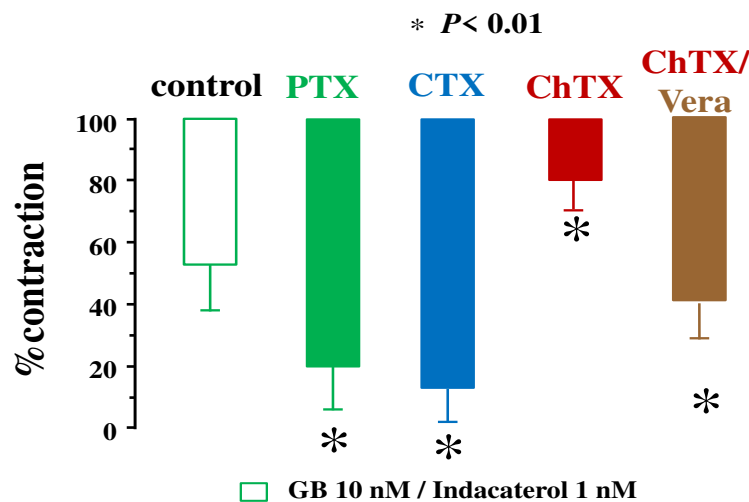
3.4. Synergistic effects between muscarinic and β_2 -adrenergic receptors

The combination of muscarinic receptor antagonists with β_2 -adrenoceptor agonists has pharmacological rationale as a bronchodilator therapy for COPD [91]. In the human airway, muscarinic contraction is more resistant to β_2 -adrenoceptor-induced relaxation than that induced by other contractile agonists [92]. Muscarinic receptors and β_2 -adrenoceptors are unevenly distributed in the human airways. β_2 -adrenoceptors were increased in the distal airways: segmental bronchus < subsegmental bronchus < lung parenchyma [93]. M_3 receptors are expressed more exclusively in segmental than subsegmental bronchus; in contrast, the M_2 subtype is widely distributed throughout the airways, while the M_1 subtype is found only in parenchyma [93]. These findings may explain why combined inhalation of a muscarinic antagonist and a β_2 -adrenoceptor agonist causes greater bronchodilation than monotherapy [94]. Furthermore, characteristic interactions between muscarinic receptors and β_2 -adrenoceptors are involved in prejunctional modulation of ACh release from parasympathetic nerve endings [95] and intracellular signaling cross-talk at the adenylyl cyclase/PKA level [96], resulting in synergistic effects on relaxation of airway smooth muscle. K_{Ca} channel activity may contribute to these interactions between these two receptors; however, little is known about the detailed underlying mechanisms.

In isometric tension recordings of guinea pig tracheal smooth muscle, indacaterol (1 nM), a long-acting β_2 -adrenoceptor agonist, modestly inhibited MCh-induced contraction (1 μ M) (Figure 4A). When glycopyrronium bromide (10 nM), a long-acting muscarinic receptor antagonist, was applied in the presence of indacaterol (1 nM), the relaxant effect of glycopyrronium bromide was significantly augmented (Figure 4A) [97]. The value of percent relaxation for the combination of indacaterol with glycopyrronium bromide was more than the sum of that for each agent. Similar results were observed between indacaterol (1 nM) and glycopyrronium (3–30 nM) [97]. Moreover, similar results were also observed between other β_2 -adrenoceptor agonists, such as salbutamol and procaterol, and other muscarinic receptor antagonists, such as atropine and tiotropium (our unpublished observation). These results indicate that the combination of muscarinic receptor antagonists with β_2 -adrenoceptor agonists causes a synergistic inhibition against muscarinic contraction. This phenomenon was observed in isolated human bronchus [98]. This synergistic effect was enhanced after exposure to pertussis toxin (1 μ g/ml) or cholera toxin (2 μ g/ml) for 6 h; in contrast, the effect was attenuated in the presence ChTX (100 nM) or IbTX (30 nM). A reduction in this synergistic effect induced by ChTX or IbTX was reversed to the control response in the presence of verapamil (Figure 4B) [99]. Inactivation of the G_i/K_{Ca} inhibitory linkage and activation of the G_s/K_{Ca} stimulatory linkage are involved in this synergistic effect between muscarinic receptor antagonists and β_2 -adrenoceptor agonists in airway smooth muscle (Figure 3) [4, 7, 53]. Moreover, the K_{Ca} /VDC channel linkage is also involved in this synergistic effect. On the other hand, synergistic effects did not occur between β_2 -adrenoceptor agonists and theophylline in airway smooth muscle (our unpublished observation). Although the clinical relevance of this result is still unknown, this result may provide evidence that combination therapy between muscarinic receptor antagonists and β_2 -adrenoceptor agonists is an effective bronchodilator therapy for COPD [100].



(A)



(B)

Figure 4. Synergistic action in combination of β_2 -adrenocetor agonists with muscarinic receptor antagonists against tracheal smooth muscle contraction. A: Left panel: A typical example of the inhibitory effects of GB (10 nM), a long-acting muscarinic receptor antagonist (LAMA) against MCh (1 μ M)-induced contraction (upper trace). A typical example of the inhibitory effects of equimolar amounts of GB in the presence of indacaterol (1 nM), a long-acting β_2 -adrenoceptor agonist (LABA) against MCh-induced contraction (1 μ M) (lower trace). Right panel: Percent inhibition of combining GB with indacaterol is greater than the sum of each agent, demonstrating synergistic action between β_2 -adrenoceptor agonists and muscarinic receptor antagonists. B: The percent inhibition of GB (10 nM) with indacaterol (1 nM) against MCh-induced contraction (1 μ M) was markedly augmented after incubation with PTX (1 μ g/ml) and CTX (2 μ g/ml) for 6 h. In contrast, the percent inhibition was significantly attenuated in the presence of ChTX (100 nM), and this ChTX-induced effect was reversed to the control level by addition of Vera (1 μ M). These results indicate that the G proteins (G_i , G_s)/ K_{Ca} channel linkage and the K_{Ca} /VDC channel linkage contributed to this synergistic action, similar to the mechanisms shown in Figures 1, 3. GB: glycopyrronium bromide, MCh: methacholine, PTX: pertussis toxin, CTX: cholera toxin, ChTX: charybdotoxin, Vera: verapamil, K_{Ca} : large-conductance Ca²⁺-activated K⁺ channels, VDC: L-type voltage-dependent Ca²⁺ channels. Cited from ref. [97, 99].

4. Airway smooth muscle tone regulated by Ca^{2+} dynamics

4.1. Membrane potential-independent Ca^{2+} dynamics

In simultaneous recordings of isometric tension and $[\text{Ca}^{2+}]_i$ in fura-2-loaded tissues of tracheal smooth muscle, various spasmogens including contractile agonists acting on GPCRs augment the tone of airway smooth muscle with elevated $[\text{Ca}^{2+}]_i$ in a concentration-dependent fashion (Figure 1) [19, 101]. However, even though contraction fully occurs, these agents cause a modest depolarization of the cell membrane in a microelectrode experiment, indicating that airway smooth muscle contracts by Ca^{2+} influx via membrane potential-independent pathways. These Ca^{2+} dynamics with a modest depolarization are involved in Ca^{2+} influx through SOC and ROC [16, 17]. Depletion of the SR Ca^{2+} stores by thapsigargin, an inhibitor of the SR Ca^{2+} -ATPase, caused an increase in $[\text{Ca}^{2+}]_i$ and contraction, demonstrating Ca^{2+} entry through SOC [17]. Because SOC was not inhibited by nifedipine, an inhibitor of VDC, VDC is not involved in SOC. Under the condition that SOC is fully activated, MCh and histamine caused further increases in $[\text{Ca}^{2+}]_i$ and tension, demonstrating Ca^{2+} entry independent of SOC and VDC (non-SOC) [17]. The Ca^{2+} influx and contraction via non-SOC was inhibited by Y-27632. In contrast, Y-27632 did not affect SOC.

4.2. Membrane potential-dependent Ca^{2+} dynamics

In fura-2-loaded tissues of tracheal smooth muscle, verapamil caused an inhibition of MCh-induced contraction with reduced $[\text{Ca}^{2+}]_i$; however, relaxant effects of verapamil are not so dramatic, indicating that VDC is partly involved in contraction mediated by GPCRs. IbTX enhanced MCh-induced contraction with elevation of $[\text{Ca}^{2+}]_i$. These effects of IbTX on tension and $[\text{Ca}^{2+}]_i$ are antagonized by verapamil [10], demonstrating that K_{Ca} channel inhibition results in contraction with elevation of $[\text{Ca}^{2+}]_i$ induced by opening VDC channels via depolarization of the cell membrane, whereas channel activation results in relaxation with reduction of $[\text{Ca}^{2+}]_i$ induced by closing VDC channels via hyperpolarization of cell membrane.

When $[\text{Ca}^{2+}]_i$ is increased by Ca^{2+} entry via various pathways described earlier (Ca^{2+} dynamics), the activity of MLCK is enhanced via CaM, leading to contraction via phosphorylation of MLC (see Section 2). In airway smooth muscle, alteration of contractility regulated by Ca^{2+} dynamics is involved in the pathophysiology implicated in asthma and COPD, such as airway limitation, airway hyperresponsiveness, and β_2 -adrenergic desensitization. It is useful to suppress Ca^{2+} dynamics for improving these pathological conditions in the airways.

4.3. Effects of Ca^{2+} release from the SR

K_{Ca} channels were activated by ACh (30 μM), substance P (0.1 μM) or IP_3 (2.4–20 μM), as well as by caffeine (5 mM), suggesting that the activity was due to Ca^{2+} released from intracellular stores. These activations with the agonists and IP_3 were markedly and reversibly reduced by heparin (50–100 $\mu\text{g/ml}$), which inhibits IP_3 binding to its receptors in the SR. Furthermore, in cultured human bronchial smooth bradykinin (0.01–1 μM), an inflammatory mediator caused bronchoconstriction and activated K_{Ca} channels in a concentration-dependent manner; the

augmented currents were inhibited by heparin (10 µg/ml) [102]. Ca²⁺ release from the SR via stimulation of IP₃ receptors causes an increase in the activation of K_{Ca} channels in smooth muscle including airways and vessels. Two pathways participate in Ca²⁺ release from the SR, the RyR pathway and the IP₃ receptor pathway. In smooth muscle cells, the IP₃ receptor is more abundant than the ryanodine receptor and reacts to IP₃, which is generated from the activation of GPCRs and phospholipase C.

5. Airway smooth muscle tone regulated by Ca²⁺ sensitization

5.1. Characteristics and physiological role of RhoA/Rho-kinase

Although an increase in [Ca²⁺]_i plays an important role in the contraction of airway smooth muscle (Figure 1) [18], it is generally considered that muscarinic receptor agonists and histamine increase tension at a constant [Ca²⁺]_i. This phenomenon is referred to as Ca²⁺ sensitization [103, 104] and is mediated by a G protein-coupled mechanism. Rho is a monomeric G protein that belongs to the Ras superfamily. The Rho family makes up a major branch that contains Rho, Rac, and Cdc42. Rho has isoforms of A-G; however, most of the function is described based on studies of RhoA. RhoA exhibits both GDP/GTP binding activity and GTPase activity, and it acts as a molecular switch between a GDP-bound inactive state (GDP-RhoA) and a GTP-bound active state (GTP-RhoA). When cells are stimulated with G protein-coupled receptor agonists, receptor tyrosine kinases and higher concentrations of potassium chloride (KCl), GDP-RhoA is converted to GTP-RhoA. RhoA and Rho-kinase are widely distributed to many organs, including the respiratory system. Rho-kinase (160 kDa) is an effector molecule of RhoA [105, 106]. Rho-kinase activated by GTP-RhoA interacts with MP and hinders MP activity by phosphorylating threonine 696 and 853 of myosin phosphatase targeting subunit 1 (MYPT1), a myosin-binding subunit [107, 108]. Rho-kinase has effects on contraction due to Ca²⁺ sensitization, stress fiber formation due to actin (cytoskeletal) reorganization, cell migration, and cell proliferation [20, 109]. These processes are implicated in the major pathophysiological characteristics of asthma and COPD, such as airflow limitation, airway hyperresponsiveness, β₂-adrenergic desensitization, eosinophil recruitment and airway remodeling [1].

5.2. Role of RhoA/Rho-kinase on contraction

Y-27632, a pyridine derivative, was developed as a specific Rho-kinase inhibitor. Y-27632 suppresses Ca²⁺ sensitization and relaxes vascular smooth muscle to treat hypertension in rats [21]. The effects of Y-27632 on MCh-induced contraction were analyzed by using strips of guinea pig airway smooth muscle treated with fura-2. Y-27632 suppressed contraction induced by agonists, such as MCh, histamine, prostaglandins, and leukotrienes, in a concentration-dependent manner, but there was no significant decrease in [Ca²⁺]_i [19]. Recently, it has been demonstrated that MYPT1 is an effective protein for Rho-kinase action on MP in airway smooth muscle cells and that Y-27632 inhibits the phosphorylation of MYPT1 in a concentration-dependent manner [108, 110]. Fasudil hydrochloride (HA-1077), a specific inhibitor of

Rho-kinase, is used clinically to suppress cerebral vasospasm following subarachnoid hemorrhage [111]. Alteration of contractility of airway smooth muscle regulated by Ca^{2+} sensitization is also involved in airflow limitation, airway hyperresponsiveness, and β_2 -adrenergic desensitization [1].

6. Role of Ca^{2+} dynamics and Ca^{2+} sensitization in airway disorders

6.1. Airflow limitation (contraction)

Airway smooth muscle contraction due to muscarinic receptor agonists (ACh, MCh and CCh), histamine, prostaglandins or leukotrienes is involved in airflow limitation, which is a characteristic feature of asthma and COPD (Figure 5). These agonists cause contraction of airway smooth muscle with increasing $[\text{Ca}^{2+}]_i$ by Ca^{2+} dynamics via Ca^{2+} entry passing through SOC, ROC, and partly VDC. Sphingosine 1-phosphate (S1P: a bioactive lysophospholipid) [108], tryptase (trypsin-like neutral serine-class protease) and SLIGKV (non-enzymatic activator of protease-activated receptor 2, PAR2) [112] released from mast cells induce airway smooth muscle contraction with increasing $[\text{Ca}^{2+}]_i$. Since clinical studies have demonstrated that S1P and tryptase may be involved in the pathophysiology of asthma, these substances have been examined as novel mediators. ATP is released from injured airway epithelium during the inflammatory processes implicated in asthma. Extracellular ATP also causes contraction of airway smooth muscle with increasing $[\text{Ca}^{2+}]_i$ [113]. Furthermore, oxidative stress and mechanical stress are related to the pathophysiology of not only COPD but also asthma. 8-isoprostaglandin $\text{F}_{2\alpha}$, an isoprostane [114], and hydrogen peroxide (H_2O_2) [84] produced by oxidative stress contract airway smooth muscle by increasing $[\text{Ca}^{2+}]_i$.

As described earlier, Y-27632 inhibited the contraction induced by spasmogens such as MCh, histamine, prostaglandins, and leukotrienes, which are involved in the pathophysiology of asthma and COPD, in a concentration-dependent manner, with no significant decrease in $[\text{Ca}^{2+}]_i$ in strips of guinea pig airway smooth muscle treated with fura-2. Furthermore, Y-27632 also inhibited the following types of contraction in a concentration-dependent manner with a modest effect on $[\text{Ca}^{2+}]_i$: contraction due to S1P and tryptase released from mast cells; contraction due to isoprostanes and Hydrogen peroxide (H_2O_2) produced by oxidative stress; and contraction due to ATP synthesized in injured airway epithelium. Spasmogens, which are implicated in the pathophysiology of asthma and COPD, cause force generation in airway smooth muscle via both Ca^{2+} influx and Ca^{2+} sensitization [115]. Force maintenance is due to Ca^{2+} sensitization induced by Rho-kinase [116]. PKC, which is an intracellular signal transduction pathway for GPCR activation, also contracts airway smooth muscle mediated by both Ca^{2+} dynamics and Ca^{2+} sensitization [22].

These findings indicate that a contractile phenotype in airway smooth muscle cells is altered by the inflammatory processes related to obstructive pulmonary diseases, such as asthma and COPD, via both Ca^{2+} dynamics and Ca^{2+} sensitization, leading to the airflow limitation (bronchoconstriction) associated with these diseases (Figure 5).

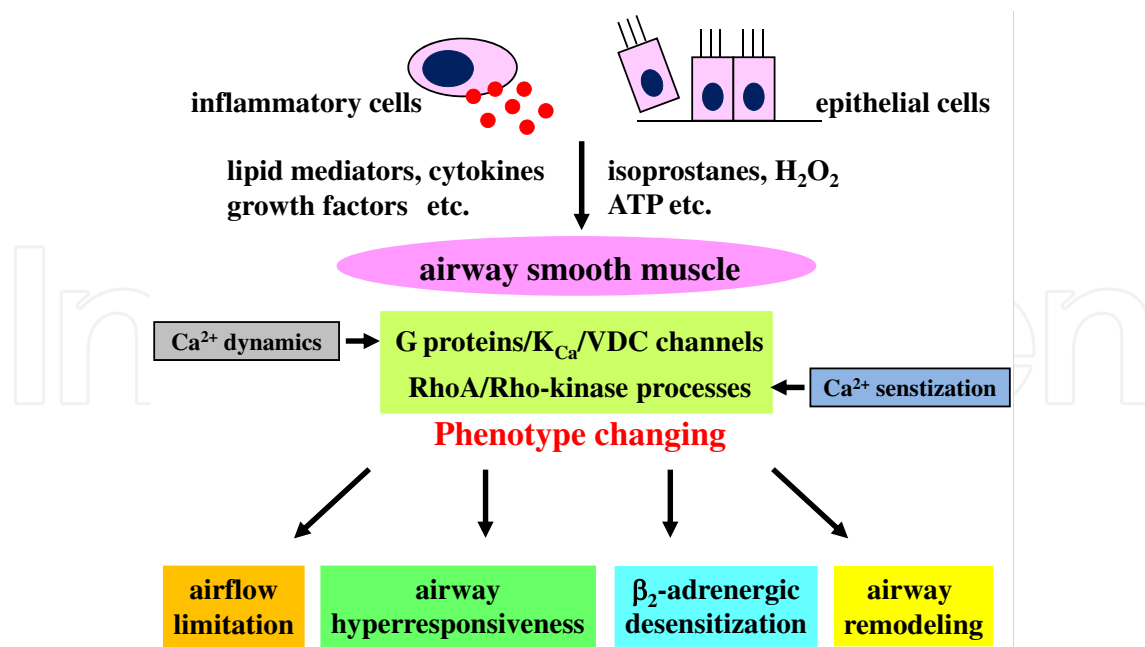


Figure 5. Involvement of G proteins/K_{Ca}/VDC channel linkage (Ca²⁺ dynamics) and RhoA/Rho-kinase processes (Ca²⁺ sensitization) in the pathophysiology of asthma and COPD. Chronic exposure to lipid mediators, cytokines, and other substances related to asthma and COPD, which are released and synthesized from inflammatory cells and epithelial cells in airways, affects airway smooth muscle functions via the G proteins/K_{Ca}/VDC channel linkage due to Ca²⁺ dynamics and RhoA/Rho-kinase processes due to Ca²⁺ sensitization. These inflammatory processes cause not only alterations of contractility but also changing to proliferative phenotype in airway smooth muscle, referred to as a phenotype change. The former phenomenon is attributed to airflow limitation, airway hyperresponsiveness, and β_2 -adrenergic desensitization; the latter phenomenon is attributed to airway remodeling via cell proliferation and migration. Therefore, G proteins/K_{Ca}/VDC channel linkage and RhoA/Rho-kinase processes are involved in almost all of the principal mechanisms of asthma and COPD. These pathways involved in Ca²⁺ dynamics and Ca²⁺ sensitization are molecular targets for therapy of these diseases. VDC: L-type voltage-dependent Ca²⁺ channels, K_{Ca}: large-conductance Ca²⁺-activated K⁺ channels. Illustrated based on ref. [1].

6.2. Airway hyperresponsiveness

Airway hyperresponsiveness is a characteristic feature of asthma, and it is essential for the diagnosis and severity assessment of asthma. Airway hyperresponsiveness is also observed in some patients with COPD. This airway disorder is clinically defined as increased responsiveness to muscarinic receptor agonists (ACh and MCh) and histamine. Airway hyperresponsiveness is mediated by various inflammatory stimulations involved in the pathophysiology of asthma, such as antigens, chemical mediators, cytokines, and eicosanoids. In a postmortem study of airway smooth muscle strips of patients with asthma, the response to histamine and ACh was greater than in healthy individuals [117]. In human airway smooth muscle passively sensitized with human asthmatic serum, contraction due to histamine is significantly elevated [118]. When airway smooth muscle is exposed for an extended period of time to interleukin (IL)-5, IL-13, IL-17, or tumor necrosis factor (TNF) $_{\alpha}$, which are released from inflammatory cells and epithelial cells in airways, contraction due to muscarinic receptor agonists and KCl is significantly increased [119, 120, 121]. This enhancement of contraction induced by TNF $_{\alpha}$ may be involved in Ca²⁺ sensitization via RhoA/Rho-kinase [110]. In the presence of a lower concentration of leukotriene C₄, KCl-induced contraction is markedly

augmented in porcine tracheal smooth muscle, and this enhanced contraction due to KCl is attenuated by Y-27632 [122]. When airway smooth muscle is exposed to S1P released from mast cells or ATP released from damaged epithelial cells, contraction in response to MCh is markedly increased after exposure to S1P or ATP, and its augmented contraction is suppressed by Y-27632 in a concentration-dependent manner [108, 113, 123]. Furthermore, pre-treatment of 8-iso-prostaglandin E₂, an isoprostane, causes an increased response to CCh in airway smooth muscle, and its augmented contraction is suppressed by Y-27632 [124]. These observations indicate that airway hyperresponsiveness is caused by direct interactions among inflammatory cells, airway epithelial cells and airway smooth muscle cells and that Ca²⁺ sensitization based on Rho-kinase-induced MYPT1 phosphorylation contributes to the airway hyperreactivity [107, 108]. Suppression of geranylgeranyltransferase, which is involved in the activation of RhoA, also reduces hyperresponsiveness in mouse bronchus [125]. Alterations of Ca²⁺ regulatory mechanisms in airway smooth muscle may play a key role in this phenomenon. Therefore, the pathophysiology of asthma (inflammatory processes involved in this disease) and alterations in the mechanical properties directly affect the function of airway smooth muscle cells via the RhoA/Rho-kinase processes. In airway smooth muscle cells, this phenotypic change for contractility induced by not only Ca²⁺ sensitization but also cytoskeleton reorganization (cell stiffness) may cause an augmented response to spasmogens [1, 126, 127]. Lung resistance in response to MCh was increased in mice sensitized by allergen challenges, as compared with control mice (airway hyperresponsiveness). Fasudil hydrochloride (HA-1077), an inhibitor of Rho-kinase, suppressed the augmented response to MCh by allergen challenges [128]. On the other hand, Ca²⁺ dynamics (Ca²⁺ mobilization) also contributes to altering the contractile phenotype of airway smooth muscle, leading to augmented responsiveness to spasmogens [129]. Moreover, acidification of esophageal lumen increases the contractile response to ACh and KCl in guinea pig trachealis mediated by activation of VDC channels and Rho-kinase [130], indicating that both Ca²⁺ dynamics and Ca²⁺ sensitization play key roles in airway hyperresponsiveness (Figure 5).

6.3. Desensitization of β_2 -adrenergic receptors

After β_2 -adrenoceptors are excessively activated, responsiveness to an agonist is attenuated. This phenomenon is referred to as desensitization of β_2 -adrenoceptors. The phosphorylation of β_2 -adrenoceptors, which leads to desensitization via uncoupling G_s from the receptors, is mediated by two types of protein kinases, cAMP-dependent PKA and cAMP-independent protein kinases such as β_2 -adrenergic receptor kinase (β ARK) [131]. PKA-induced phosphorylation, which is produced by exposure to a low concentration of β_2 -adrenoceptor agonists, leads to heterologous desensitization (a nonspecific reduced response to other agonists involving cAMP) [132]. On the other hand, β ARK-induced phosphorylation, which is produced by exposure to a high concentration of β_2 -adrenoceptor agonists, leads to homologous desensitization (a specific reduced response to β_2 -adrenoceptor agonist) [133]. These phenomena also occur in tracheal smooth muscle, including human tissues [10, 134, 135, 136]. β_2 -adrenergic desensitization occurs after continuous [134, 135, 136] or repetitive administration [10, 135, 136] of β_2 -adrenoceptor agonists or after exposure to substances related to the inflammatory processes in asthma, including inflammatory cytokines such as IL-1 β [137],

growth factors such as transforming growth factor (TGF)- β 1 [138] and platelet-derived growth factor (PDGF) [139], lipid mediators such as lysophosphatidylcholine (Lyso-PC), a lysophospholipid produced by phospholipase A2 [140], and S1P [141], or PAR2 agonists such as tryptase and SLIGKV [112]. Therefore, desensitization of β_2 -adrenoceptors in airway smooth muscle is an extremely important phenomenon that occurs due to both the treatment and the pathophysiology of asthma. Reduced responsiveness to β_2 -adrenoceptor agonists after excessive or repeated exposure to these agonists was prevented when G_s linked to β_2 -adrenoceptors was irreversibly activated by pre-treating airway smooth muscle with cholera toxin (2 μ g/ml) for 6 h [134, 135, 142, 143] (Figure 6A). On the other hand, in the presence of ChTX or IbTX, this β_2 -adrenergic desensitization was markedly enhanced [134, 135]. Inactivation of the G_s/K_{Ca} channel linkage plays an important role in β_2 -adrenergic desensitization (Figures 5, 8).

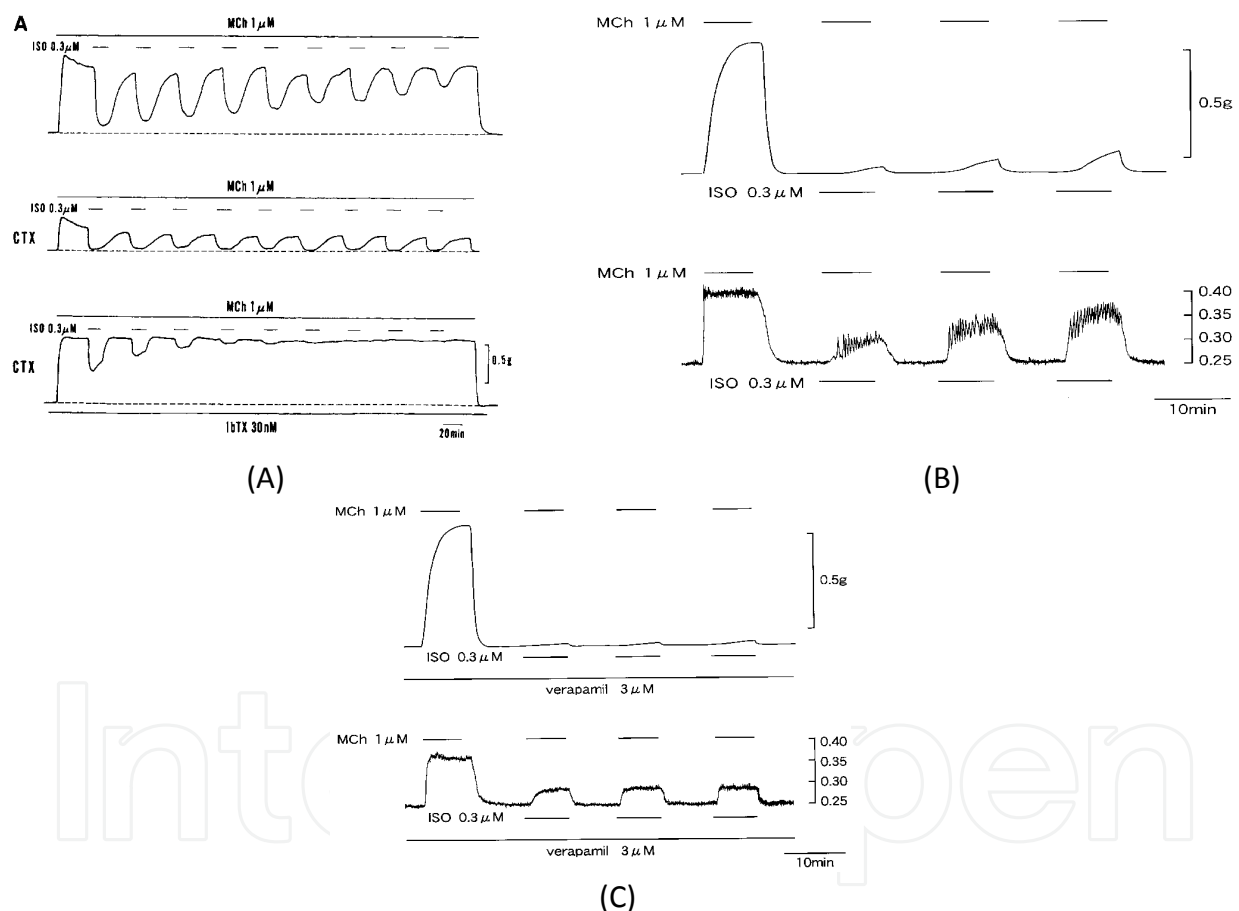


Figure 6. Inhibitory effects of G_s/K_{Ca} channel linkage on the β -adrenergic desensitization after repeated exposure to a β -adrenoceptor agonist in isometric tension recording of tracheal smooth muscle. (A) A typical example of repeated application of ISO (0.3 μ M) to tissues precontracted by MCh (1 μ M) at intervals of 20 min under the following experimental conditions: control (upper trace), preincubation with CTX (2 μ g/ml) for 6 h (middle trace), and preincubation with CTX and in the presence of IbTX (30 nM) throughout the experiment (lower trace). The G_s/K_{Ca} channel stimulatory linkage is involved in the prevention of β_2 -adrenergic desensitization. (B) A typical example of simultaneously recorded isometric tension (upper trace) and F_{340}/F_{380} (lower trace) after repeated exposure to MCh (1 μ M) with ISO (0.3 μ M) in fura-2-loaded tissues of tracheal smooth muscle in guinea pigs. (C) A typical example of simultaneously recorded isometric tension (upper trace) and F_{340}/F_{380} (lower trace) after repeated exposure to MCh (1

μM) with ISO (0.3 μM) in the presence of verapamil (3 μM) in fura-2-loaded tissues similar to (B). Ca^{2+} dynamics via the K_{Ca} /VDC channel linkage are involved in β_2 -adrenergic desensitization. ISO: isoprenaline, MCh: methacholine, CTX: cholera toxin, IbTX: iberiotoxin, K_{Ca} channels: large-conductance Ca^{2+} -activated K^+ channels. Cited from ref. [10, 135].

6.3.1. Ca^{2+} dynamics

In fura-2-loaded tissues of guinea pig tracheal smooth muscle, the relaxant effect of isoprenaline on MCh-induced contraction was gradually attenuated with increasing $[\text{Ca}^{2+}]_{\text{i}}$ following repeated exposure to isoprenaline with MCh for 10 min every 30 min [10, 135] (Figure 6B), and this reduced responsiveness to isoprenaline was avoided by pre-exposure to cholera toxin or the addition of verapamil with no change in $[\text{Ca}^{2+}]_{\text{i}}$ [10] (Figure 6C). In contrast, after repeated exposure to forskolin, db-cAMP and theophylline, the relaxant effect of these cAMP-related agents was not diminished with no change in $[\text{Ca}^{2+}]_{\text{i}}$ (homologous desensitization) [10, 135]. Furthermore, after exposure to PDGF for 15 min, the relaxant effect of isoprenaline against MCh-induced contraction was markedly attenuated with increasing $[\text{Ca}^{2+}]_{\text{i}}$, and this reduced responsiveness to isoprenaline was reversed by verapamil [139]. The relaxant effects of not only β_2 -adrenoceptor agonists but also forskolin are markedly attenuated with elevated $[\text{Ca}^{2+}]_{\text{i}}$ after exposure to growth factors, such as $\text{TGF}\beta_1$ and PDGF (heterologous desensitization) (Figure 8). In contrast, the relaxant effects of db-cAMP and theophylline are not diminished after exposure to $\text{TGF}\beta_1$ and PDGF. These results indicate that β_2 -adrenergic desensitization occurs via dysfunction of the receptor/ G_s /adenylyl cyclase processes in airway smooth muscle and that the cAMP-independent pathway is involved in this phenomenon [3, 4, 7, 8]. These results indicate that the Ca^{2+} influx passing through VDC is involved in β_2 -adrenergic desensitization and that VDC activity may be augmented by dysfunction of the $\text{G}_s/\text{K}_{\text{Ca}}$ channel stimulatory linkage (Figures 5, 8).

6.3.2. Ca^{2+} sensitization

In fura-2-loaded tissues of guinea pig tracheal smooth muscle, the inhibitory effect of isoprenaline against MCh-induced contraction following continuous exposure to Lyso-PC [140] was markedly attenuated with no changes in $[\text{Ca}^{2+}]_{\text{i}}$ (Figure 7A). This reduced responsiveness to isoprenaline was reversed to the control response by application of Y-27632 in a concentration-dependent manner (Figure 7B). In contrast, the relaxant effect of cAMP-related agents such as forskolin, theophylline, and db-cAMP, was not diminished after exposure to Lyso-PC (homologous desensitization). Similar to Lyso-PC, reduced responsiveness to isoprenaline was observed with no changes in $[\text{Ca}^{2+}]_{\text{i}}$ after the exposure of tracheal smooth muscle to tryptase and SLIGKV [112] and S1P [141]. The relaxant effects of forskolin were not attenuated after exposure to tryptase and SLIGKV; in contrast, the relaxant effects were markedly diminished after exposure to S1P, indicating that the receptor/ G_s /adenylyl cyclase process is also involved in the dysfunction of β_2 -adrenoceptors in airway smooth muscle. cAMP activity may still be intact under this condition of excessive stimulation of β_2 -adrenoceptors. Furthermore, in the presence of bisindolylmaleimide, a membrane-permeable inhibitor of PKC, reduced responsiveness to isoprenaline is not prevented after exposure to an agonist [134, 135, 140]. These observations indicate that after exposure to these lipid mediators and PAR2 agonists, tolerance

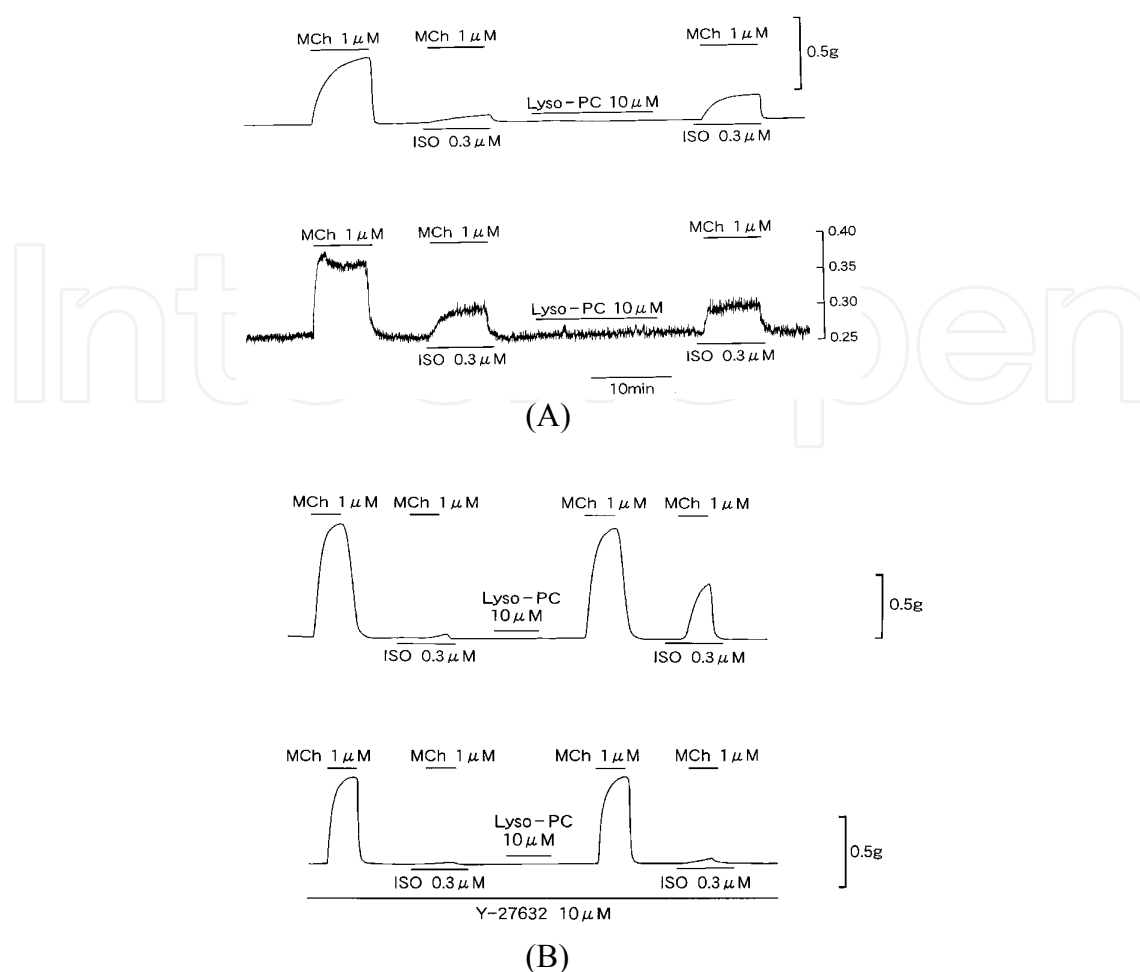


Figure 7. The effects of Ca²⁺ sensitization mediated by RhoA/Rho-kinase on β_2 -adrenergic desensitization in tracheal smooth muscle. A: A typical example of simultaneously recorded isometric tension (upper trace) and F_{340}/F_{380} ratio (lower trace) induced by MCh (1 μ M) with ISO (0.3 μ M) inhibition before and after exposure to Lyso-PC (10 μ M) for 15 min. Pretreatment with Lyso-PC attenuates ISO-induced relaxation without elevating $[Ca^{2+}]_i$, indicating that Ca²⁺ sensitization is involved in β_2 -adrenergic desensitization. B: A typical example of the inhibitory effects of ISO (0.3 μ M) on MCh-induced contraction (1 μ M) before and after exposure to Lyso-PC (10 μ M) for 15 min in the absence (upper trace) and presence (lower trace) of Y-27632 (10 μ M) throughout the experiments. Y-27632 inhibits β_2 -adrenergic desensitization induced by Lyso-PC, indicating that Ca²⁺ sensitization via RhoA/Rho-kinase processes is involved in this phenomenon. MCh: methacholine, ISO: isoprenaline, Lyso-PC: lysophosphatidylcholine. Cited from ref. [140].

to β_2 -adrenoceptor agonists occurs due to Ca²⁺ sensitization via the RhoA/Rho-kinase processes, not via PKC. This β_2 -adrenergic desensitization is caused by elevated sensitization to intracellular Ca²⁺ based on G_s inactivation and Rho-kinase activation, although little is known about the functional relationship between G_s and RhoA/Rho-kinase (Figures 5, 8).

6.3.3. Intrinsic efficacy

The potency of a β_2 -adrenoceptor agonist depends on its receptor affinity and intrinsic efficacy. Intrinsic efficacy (intrinsic activity) refers to the ability of an agent to activate its receptors without regard for their concentration. Some agonists completely activate receptors, but others only partially activate them. The former are referred to as full agonists, and the latter are

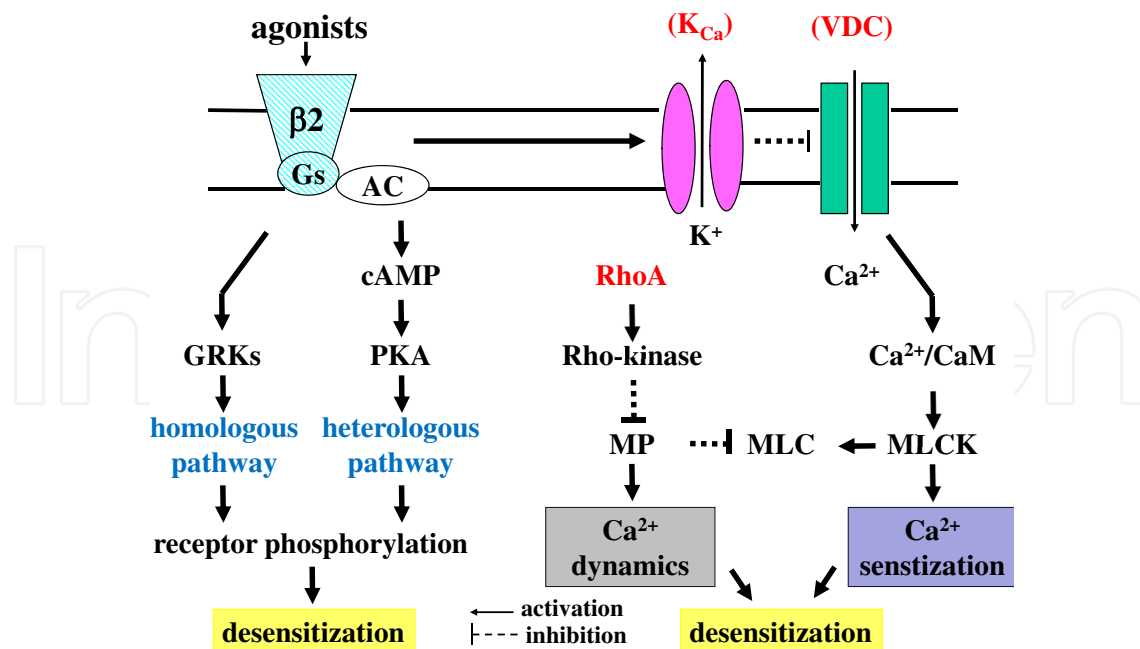


Figure 8. Role of Ca^{2+} dynamics and Ca^{2+} sensitization in the desensitization of β_2 -adrenoceptors in airway smooth muscle. Phosphorylation of β_2 -adrenoceptors is essential for reduced responsiveness to their agonists. There are two pathways in the mechanisms of β_2 -adrenergic desensitization: 1) cAMP-independent phosphorylation of their receptors via members of the GRK family such as β ARK (homologous desensitization), and 2) cAMP-dependent phosphorylation of their receptors via PKA (heterologous desensitization). Inactivation of G_s , which is linked to β_2 -adrenoceptors, is involved in desensitization of the receptors mediated by Ca^{2+} dynamics and Ca^{2+} sensitization. Impairment of the stimulatory linkage between G_s /PKA and K_{Ca} channels causes an increase in the membrane potential, leading to Ca^{2+} influx passing through VDC channels (Ca^{2+} dynamics: Ca^{2+} -dependent mechanisms). On the other hand, impairment of the inhibitory correlation between G_s /PKA and RhoA/Rho-kinase processes causes an increase in Rho-kinase activity, leading to a reduced MP activity (Ca^{2+} sensitization: Ca^{2+} -independent mechanisms). β_2 : β_2 -adrenoceptors, AC: adenylyl cyclase, GRK: G protein-receptor kinase, β ARK: β -adrenoceptor kinase, PKA: protein kinase A, MLCK: myosin light chain kinase, MLC: myosin light chain, MP: myosin phosphatase, K_{Ca} : large-conductance Ca^{2+} -activated K^+ channels, VDC: L-type voltage-dependent Ca^{2+} channels. Illustrated based on ref. [1, 2, 10, 112, 134, 135, 136, 138, 139, 140, 141, 142, 145, 146].

referred to as partial agonists. Moreover, partial agonists are subclassified as weak partial agonists, which have lower efficacy, and strong partial agonists, which have higher efficacy [144, 145]. Intrinsic efficacy was measured indirectly as a physiological response (changes in smooth muscle relaxation determined by isometric tension recording *in vitro*) [145]. The ratio of the intrinsic efficacy of any two β_2 -agonists is expressed as a fraction between 0 and 1 by concentration-inhibition curves, taking that of adrenaline as 1. The order of efficacy (the maximal percent relaxation against 10 μM MCh-induced contraction) was as follows: isoprenaline = adrenaline > indacaterol, formoterol, procaterol > salbutamol > salmeterol > tulobutanol [97, 145] (Table 1); these efficacies are similar to the values measured by changing the level of intracellular cAMP [144]. Isoprenaline behaves as a full agonist, and other agonists behave as partial agonists. Isoprenaline caused β_2 -adrenergic desensitization greater than that of other agonists, indicating that excessive activation of a full agonist leads to reduced responsiveness to β_2 -adrenoceptor agonists in airway smooth muscle [134, 135, 136, 142, 145]. In contrast, tulobutanol, which is the weakest partial agonist, caused a modest reduction in response to an agonist, even in cases of excessive exposure to tulobutanol [146].

β_2 -adrenoreceptor agonists	The maximal inhibition		classification
	(1 μ M MCh)	(10 μ M MCh)	
isoprenaline	100.0	100.0	Full agonists
adrenaline	100.0	100.0	
indacaterol	100.0	72.3	Strong partial agonists
formoterol	100.0	68.9	
procaterol	100.0	66.8	
salbutamol	78.1	48.2	Weak partial agonists
salmeterol	59.4	32.1	
tulobuterol	34.6	20.6	
propranolol	0	0	antagonists

Table 1. Intrinsic efficacy of β_2 -adrenoceptor agonists. Values of intrinsic efficacy of β_2 -adrenoceptor agonists were measured as a physiologic response in airway smooth muscle. The values of intrinsic efficacy were expressed as the maximum percent inhibition for each β_2 -adrenoceptor agonist against MCh-induced contraction (1 and 10 μ M) in guinea pig tracheal smooth muscle. MCh: methacholine. Cited from ref. [1, 97, 145].

6.4. Airway remodeling

Airway inflammatory reactions involving activated eosinophils act on the epithelium, subepithelium, and smooth muscle layers and bring about characteristic structural changes in the airways. Subepithelial fibrosis results from the deposition of collagen fibers and proteoglycans under the basement membrane (thickening of the airway wall). This phenomenon is known as airway remodeling, which is thought to be related to asthma severity. Airway smooth muscle contributes to airway remodeling by mass formation via cell proliferation and migration [147, 148]. Unlike normal cells, increased airway smooth muscle cell proliferation in patients with asthma is not suppressed by glucocorticosteroids because of CCAAT/enhancer-binding protein (C/EBP)- α deficiency in airway smooth muscle cells [149].

6.4.1. Cell proliferation

Factors facilitating the proliferation of airway smooth muscle cells are roughly divided into the following two groups: 1) ligands (polypeptide growth factors) of tyrosine kinase receptors (RTKs), such as epidermal growth factor (EGF) and PDGF, and 2) ligands (contractile agents) of GPCRs, such as leukotriene D₄, thromboxane A₂ and endothelin. When ligands bind to growth factor receptors, tyrosine kinase is first activated, followed by Ras and extracellular regulated kinase (ERK)1/2, to transmit information to the nucleus [150]. Next, via cyclin D1 activation, DNA synthesis and cell proliferation occur [151]. In addition to this main pathway for smooth muscle proliferation, cross-talk between RTKs and GPCRs is mediated by phos-

phatidylinositol 3-kinase (PI3K), p70S6 kinase, and glycogen synthase kinase-3 (GSK-3) [150, 152]. The involvement of the Rho family (RhoA, Rac and Cdc42) in the control mechanisms of airway smooth muscle cell proliferation has not been sufficiently clarified. EGF- and PDGF-induced cell proliferation is not suppressed by inactivation of RhoA/Rho-kinase signaling [126]; in contrast, the activation of RhoA, not Rac or cdc42, causes the proliferation of human bronchial smooth muscle cells that have been stimulated with serum. This proliferative reaction is suppressed by Y-27632, C3 exoenzyme, and simvastatin, a HMG-CoA reductase inhibitor, which attenuate proliferation via the geranylgeranylation of RhoA [153]. Another factor, M₂ muscarinic receptor, facilitates the proliferation of airway smooth muscle cells [154, 155]. A recent clinical trial has demonstrated that an antagonist of VDC channels inhibits airway remodeling in patients with severe asthma [156]. Therefore, Ca²⁺ influx via VDC channels is enhanced since K_{Ca} channel activity is attenuated by G_i when MCh is applied to airway smooth muscle [7, 8]. These results indicate that both Ca²⁺ dynamics and Ca²⁺ sensitization contribute to the proliferation of airway smooth muscle cells (Figure 5).

6.4.2. Cell migration

Cell migration is a characteristic function of inflammatory cells, fibroblasts and smooth muscle cells, and it plays an important role in various pathophysiological environments, such as inflammatory cell infiltration and airway smooth muscle hyperplasia [157]. Migration of airway smooth muscle cells is enhanced by the extracellular matrix [158]. Cell migration occurs due to contraction involving actin, myosin reactions and actin reorganization. Since RhoA/Rho-kinase signaling is the most important factor controlling the cytoskeleton of airway smooth muscle cells and other cells [159], this pathway may control the migration of airway smooth muscle cells via changes in the cytoskeleton. Hence, RhoA/Rho-kinase may be involved in airway remodeling mediated not only by cell proliferation but also by cell migration. Urokinase, PDGF, leukotriene and lysophosphatidic acid facilitate the migration of human airway smooth muscle cells [160, 161, 162, 163]. Moreover, heat shock protein, PI3K, p38 mitogen-activated protein kinase, prostaglandin D₂, and IL-13 facilitate airway smooth muscle migration [160, 164, 165]. Y-27632 significantly suppresses the increased migration of airway smooth muscle cells, due to PDGF or leukotriene stimulation [161, 162], indicating that RhoA/Rho-kinase signaling (Ca²⁺ sensitization) plays an important role in controlling cell migration (Figure 5). On the other hand, Ca²⁺ dynamics regulate the migration of airway smooth muscle cells and inflammatory cells. Ca²⁺ influx via SOC channels contributes to PDGF-induced cell migration of airway smooth muscle [166], and increasing [Ca²⁺]_i via other mechanisms also causes substance P-induced cell migration of airway smooth muscle [167] (Figure 5). Since IL-13 enhances Ca²⁺ oscillation in airway smooth muscle cells, cell migration induced by IL-13 may be regulated by Ca²⁺ dynamics [168].

6.4.3. Interaction between airway smooth muscle and inflammatory cells

As described earlier, contractility of airway smooth muscle is altered by tryptase and S1P, which are released from mast cells, and Lyso-PC, which is synthesized in the membrane of various inflammatory cells [108, 112, 140, 141]. Ca²⁺ sensitization by RhoA/Rho-kinase

processes contributes to this phenomenon. When sensitized mice are subjected to allergen challenges, eosinophil infiltration is markedly increased in the airways. In allergen-challenged mice, pretreatment with Rho-kinase inhibitors such as Y-27632 or fasudil hydrochloride (HA-1077) markedly suppressed an increase in eosinophil recruitment in the airway in a dose-dependent manner [128]. The actions of Lyso-PC are mediated by RhoA/Rho-kinase, leading to β_2 -adrenergic desensitization [140], and administration of Lyso-PC to guinea pigs enhances eosinophil recruitment and resistance in the airways [169]. The effects of S1P are also mediated by RhoA/Rho-kinase processes, leading to airway hyperresponsiveness [108] and remodeling [170]. S1P increased mRNA and protein expression of vascular cell adhesion molecule (VCAM)-1 when S1P is applied to pulmonary endothelial cells, leading to eosinophil infiltration to the airways, and this upregulation of VCAM-1 is attenuated by C3 exoenzyme and Y-27632 [171]. Y-27632 reduces not only the number of eosinophils but also macrophages and neutrophils in an animal model of allergic asthma [172]. Ca²⁺ sensitization via RhoA/Rho-kinase processes contributes to recruitment of inflammatory cells to the airways.

Therefore, Ca²⁺ sensitization by RhoA/Rho-kinase processes [1, 173, 174, 175] and Ca²⁺ dynamics by ion channels including VDC and SOC [6, 11, 12, 176] may be a therapeutic target for obstructive pulmonary diseases including asthma.

7. Conclusions

Ca²⁺ signaling, which is due to Ca²⁺ dynamics and Ca²⁺ sensitization, contributes to alterations of contractility that lead to airway disorders (airflow limitation, airway hyperresponsiveness, and β_2 -adrenergic desensitization), which are characteristic features of asthma and COPD. Ca²⁺ dynamics and Ca²⁺ sensitization also facilitate the proliferation and migration of airway smooth muscle via changing to proliferative phenotype. A recent report has indicated that bitter taste receptor stimulation causes relaxation of airway smooth muscle via activation of K_{Ca} channels [177]. Hence, Ca²⁺ dynamics due to G proteins/K_{Ca}/VDC channels and Ca²⁺ sensitization due to RhoA/Rho-kinase processes may be therapeutic targets for asthma and COPD, and research in these areas may provide novel strategies in the development of bronchodilators for these diseases.

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References

- [1] Kume H. RhoA/Rho-kinase as a therapeutic target in asthma. *Curr Med Chem* 2008;15:2876-85.
- [2] Oguma T, Kume H, Ito S, Takeda N, Honjo H, Kodama I, Shimokata K, Kamiya K. Involvement of reduced sensitivity to Ca^{2+} in β -adrenergic action on airway smooth muscle. *Clin Exp Allergy* 2006;36:183-91.
- [3] Kume H, Takai A, Tokuno H, Tomita T. Regulation of Ca^{2+} -dependent K^{+} -channel activity in tracheal myocytes by phosphorylation. *Nature* 1989;341:152-4.
- [4] Kume H, Hall IP, Washabau RJ, Takagi K, Kotlikoff MI. β -adrenergic agonists regulate K_{Ca} channels in airway smooth muscle by cAMP-dependent and -independent mechanisms. *J Clin Invest* 1994;93:371-9.
- [5] Tomita T, Kume H. Electrophysiology of potassium channels in airways smooth muscle. In: Raeburn D, Giembycz MA (ed.) *Airways Smooth Muscle: Development and Regulation of Contractility*. Birkhauser Verlag, Basel, 1994,pp.163-184.
- [6] Kume H. Large-conductance calcium-activated potassium channels. In: Wang YX (ed.) *Calcium Signaling in Airway Smooth Muscle Cells*. Springer, New York, 2013,pp.49-83.
- [7] Kume H, Graziano MP, Kotlikoff MI. Stimulatory and inhibitory regulation of calcium-activated potassium channels by guanine nucleotide-binding proteins. *Proc Natl Acad Sci USA* 1992;89:11051-5.
- [8] Kume H, Kotlikoff MI. Muscarinic inhibition of single K_{Ca} channels in smooth muscle cells by a pertussis-sensitive G protein. *Am J Physiol* 1991;261:C1204-9
- [9] Kume H, Takagi K, Satake T, Tokuno H, Tomita T. Effects of intracellular pH on calcium-activated potassium channels in rabbit tracheal smooth muscle. *J Physiol* 1990;424:445-57.
- [10] Kume H, Ishikawa T, Oguma T, Ito S, Shimokata K, Kotlikoff MI. Involvement of Ca^{2+} mobilization in tachyphylaxis to β -adrenergic receptors in trachealis. *Am J Respir Cell Mol Biol* 2003;29:359-66.
- [11] Mahn K, Ojo OO, Chadwick G, Aaronson PI, Ward JP, Lee TH. Ca^{2+} homeostasis and structural and functional remodelling of airway smooth muscle in asthma. *Thorax* 2010;65:547-52.
- [12] Koopmans T, Anaparti V, Castro-Piedras I, Yarova P, Irechukwu N, Nelson C, Perez-Zoghbi J, Tan X, Ward JP, Wright DB. Ca^{2+} handling and sensitivity in airway smooth muscle: emerging concepts for mechanistic understanding and therapeutic targeting. *Pulm Pharmacol Ther* 2014;29:108-20.

- [13] Halayko AJ, Tran T, Gosens R. Phenotype and functional plasticity of airway smooth muscle: role of caveolae and caveolins. *Proc Am Thorac Soc* 2008;5:80-8.
- [14] Wright DB, Trian T, Siddiqui S, Pascoe CD, Johnson JR, Dekkers BG, Dakshinamurti S, Bagchi R, Burgess JK, Kanabar V, Ojo OO. Phenotype modulation of airway smooth muscle in asthma. *Pulm Pharmacol Ther* 2013;26:42-9.
- [15] Schaafsma D, Gosens R, Bos IS, Meurs H, Zaagsma J, Nelemans SA. Allergic sensitization enhances the contribution of Rho-kinase to airway smooth muscle contraction. *Br J Pharmacol* 2004; 143: 477-84.
- [16] Murray RK, Kotlikoff MI. Receptor-activated calcium influx in human airway smooth muscle cells. *J Physiol* 1991;435:123-44.
- [17] Ito S, Kume H, Yamaki K, Katoh H, Honjo H, Kodama I, Hayashi H. Regulation of capacitative and noncapacitative receptor-operated Ca²⁺ entry by rho-kinase in tracheal smooth muscle. *Am J Respir Cell Mol Biol* 2002;26:491-8.
- [18] Gerthoffer WT. Regulation of the contractile element of airway smooth muscle. *Am J Physiol* 1991;261:L15-28.
- [19] Ito S, Kume H, Honjo H, Katoh H, Kodama I, Yamaki K, Hayashi H. Possible involvement of Rho kinase in Ca²⁺ sensitization and mobilization by MCh in tracheal smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L1218-24.
- [20] Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 1996;273:245-8.
- [21] Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997;389:990-4.
- [22] Mukherjee S, Trice J, Shinde P, Willis RE, Pressley TA, Perez-Zoghbi JF. Ca²⁺ oscillations, Ca²⁺ sensitization, and contraction activated by protein kinase C in small airway smooth muscle. *J Gen Physiol* 2013;141:165-78.
- [23] Yoshii A, Iizuka K, Dobashi K, Horie T, Harada T, Nakazawa T, Mori M. Relaxation of contracted rabbit tracheal and human bronchial smooth muscle by Y-27632 through inhibition of Ca²⁺ sensitization. *Am J Respir Cell Mol Biol* 1999;20:190-200.
- [24] Atkinson NS, Robertson GA, Ganetzky B. A component of calcium-activated potassium channels encoded by the *Drosophila* slo locus. *Science* 1991;253:551-5.
- [25] Butler A, Tsunoda S, McCobb DP, Wei A, Salkoff L. mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels. *Science* 1993;261:221-4.
- [26] Wallner M, Meera P, Toro L. Determinant for beta-subunit regulation in high-conductance voltage-activated and Ca²⁺-sensitive K⁺ channels: an additional transmembrane region at the N terminus. *Proc Natl Acad Sci USA* 1996;93:14922-7.

- [27] Jiang Y, Pico A, Cadene M, Chait BT, MacKinnon R. Structure of the RCK domain from the *E. coli* K⁺ channel and demonstration of its presence in the human BK channel. *Neuron* 2001;29:593-601.
- [28] Shi J, Krishnamoorthy G, Yang Y, Hu L, Chaturvedi N, Harilal D, Qin J, Cui J. Mechanism of magnesium activation of calcium-activated potassium channels. *Nature* 2002;418:876-80.
- [29] Park JK, Kim YC, Sim JH, Choi MY, Choi W, Hwang KK, Cho MC, Kim KW, Lim SW, Lee SJ. Regulation of membrane excitability by intracellular pH (pHi) changers through Ca²⁺-activated K⁺ current (BK channel) in single smooth muscle cells from rabbit basilar artery. *Pflügers Arch* 2007;454:307-19.
- [30] Knaus HG, Folander K, Garcia-Calvo M, Garcia ML, Kaczorowski GJ, Smith M, Swanson R. Primary sequence and immunological characterization of β -subunit of high conductance Ca²⁺-activated K⁺ channel from smooth muscle. *J Biol Chem* 1994;269:17274-8.
- [31] McCann JD, Welsh MJ. Calcium-activated potassium channels in canine airway smooth muscle. *J Physiol* 1986;372:113-127.
- [32] Green KA, Foster RW, Small RC. A patch-clamp study of K⁺ channel activity in bovine isolated tracheal smooth muscle cells. *Br J Pharmacol* 1991;102:871-8.
- [33] Saunders HH, Farley JM. Pharmacological properties of potassium currents in swine tracheal smooth muscle. *J Pharmacol Exp Ther* 1992;260:1038-1044.
- [34] Snetkov VA, Hirst SJ, Twort CH, Ward JP. Potassium currents in human freshly isolated bronchial smooth muscle cells. *Br J Pharmacol* 1995;115:1117-25.
- [35] Snetkov VA, Hirst SJ, Ward JP. Ion channels in freshly isolated and cultured human bronchial smooth muscle cells. *Exp Physiol* 1996;81:791-804.
- [36] Lattorre R, Oberhauser A, Labarca P, Alvarez O. Varieties of calcium-activated potassium channels. *Annu Rev Physiol* 1989;51:385-399.
- [37] Trieschmann U, Isenberg G. Ca²⁺-activated K⁺ channels contribute to the resting potential of vascular myocytes. Ca²⁺-sensitivity is increased by intracellular Mg²⁺-ions. *Pflügers Arch* 1989;414:S183-4.
- [38] Murray MA, Berry JL, Cook SJ, Foster RW, Green KA, Small RC. Guinea-pig isolated trachealis; the effects of charybdotoxin on mechanical activity, membrane potential changes and the activity of plasmalemmal K⁺-channels. *Br J Pharmacol* 1991;103:1814-1818.
- [39] Kirkpatrick CT. Tracheobronchial smooth muscle. In: Bulbrung E, Brading AE, Jones AW, Tomita T (ed.) *Smooth Muscle; An Assessment of Current Knowledge*. Edward Arnold, London, 1981, pp.385-95.

- [40] Isaac L, McArdle S, Miller NM, Foster RW, Small RC. Effects of some K⁺-channel inhibitors on the electrical behaviour of guinea-pig isolated trachealis and on its responses to spasmogenic drugs. *Br J Pharmacol* 1996;117:1653-62.
- [41] Berkefeld H, Fakler B, Schulte U. Ca²⁺-activated K⁺ channels: from protein complexes to function. *Physiol Rev* 2010;90:1437-59.
- [42] Hu XQ, Zhang L. Function and regulation of large conductance Ca²⁺-activated K⁺ channel in vascular smooth muscle cells. *Drug Discov Today* 2012;17:974-87.
- [43] Semenov I, Wang B, Herlihy JT, Brenner R. BK channel β 1-subunit regulation of calcium handling and constriction in tracheal smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L802-10.
- [44] Nara M, Dhulipala PD, Wang YX, Kotlikoff MI. Reconstitution of β -adrenergic modulation of large conductance, calcium-activated potassium (maxi-K) channels in *Xenopus* oocytes. Identification of the cAMP-dependent protein kinase phosphorylation site. *J Biol Chem* 1998;273:14920-4.
- [45] Hiramatsu T, Kume H, Kotlikoff MI, Takagi K. Role of calcium-activated potassium channels in the relaxation of tracheal smooth muscles by forskolin. *Clin Exp Pharmacol Physiol* 1994;21:367-75.
- [46] Sadoshima J, Akaike N, Kanaide H, Nakamura M. Cyclic AMP modulates Ca²⁺-activated K⁺ channel in cultured smooth muscle cells of rat aortas. *Am J Physiol* 1988;255:H754-9.
- [47] Toro L, Stefani E, Erulkar S. Hormonal regulation of potassium current in single myometrium cells. *Proc Natl Acad Sci USA* 1990;87:2892-5.
- [48] Magleby KL, Pallotta BS. Calcium dependence of open and shut interval distributions from calcium-activated potassium channels in cultured rat muscle. *J Physiol (Lond)* 1983;344:585-604.
- [49] Honda K, Satake T, Takagi K, Tomita T. Effects of relaxants on electrical and mechanical activities in the guinea-pig tracheal muscle. *Br J Pharmacol* 1986;87:665-71.
- [50] Honda K, Tomita T. Electrical activity in isolated human tracheal muscle. *Jpn J Physiol* 1987;37:333-6.
- [51] Jones TR, Charette L, Garcia ML, Kaczorowski GJ. Selective inhibition of relaxation of guinea-pig trachea by charybdotoxin, a potent Ca²⁺-activated K⁺ channel inhibitor. *J Pharmacol Exp Ther* 1990;255:697-706.
- [52] Miura M, Belvisi MG, Stretton CD, Yacoub MH, Barnes PJ. Role of potassium channels in bronchodilator responses in human airways. *Am Rev Respir Dis* 1992;146:132-6.

- [53] Kume H, Mikawa K, Takagi K, Kotlikoff MI. Role of G proteins and K_{Ca} channels in the muscarinic and β -adrenergic regulation of airway smooth muscle. *Am J Physiol* 1995;268:L221–9.
- [54] Wang YX, Fleischmann BK, Kotlikoff MI. Modulation of maxi- K^+ channels by voltage-dependent Ca^{2+} channels and methacholine in single airway myocytes. *Am J Physiol* 1997;272:C1151–9.
- [55] Hisada T, Kurachi Y, Sugimoto T. Properties of membrane currents in isolated smooth muscle from guinea-pig trachea. *Pflugers Arch* 1990; 416: 151–61.
- [56] Saunders HH, Farley JM. Pharmacological properties of potassium currents in swine tracheal smooth muscle. *J Pharmacol Exp Ther* 1992;260:1038–44.
- [57] Zhou XB, Wulfsen I, Lutz S, Utku E, Sausbier U, Ruth P, Wieland T, Korth M. M_2 muscarinic receptors induce airway smooth muscle activation via a dual, $G\beta\gamma$ -mediated inhibition of large conductance Ca^{2+} -activated K^+ channel activity. *J Biol Chem* 2008;283:21036–44.
- [58] Semenov I, Wang B, Herlihy JT, Brenner R. BK channel β_1 subunits regulate airway contraction secondary to M_2 muscarinic acetylcholine receptor mediated depolarization. *J Physiol* 2011;589:1803–17.
- [59] Tare M, Parkington HC, Coleman HA, Neild TO, Dusting GJ. Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature* 1990;346: 69–71.
- [60] Mitchell JA, Ali F, Bailey L, Moreno L, Harrington LS. Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium. *Exp Physiol* 2008;9:141–7.
- [61] Archer SL, Huang JM, Hampl V, Nelson DP, Shultz PJ, Weir EK. Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc Natl Acad Sci USA* 1994; 91:7583–7.
- [62] Mikawa K, Kume H, Takagi K. Effects of BK_{Ca} channels on the reduction of cytosolic Ca^{2+} in cGMP-induced relaxation of guinea-pig trachea. *Clin Exp Pharmacol Physiol* 1997;24:175–81.
- [63] Nara M, Dhulipala PD, Ji GJ, Kamasani UR, Wang YX, Matalon S, Kotlikoff MI. Guanylyl cyclase stimulatory coupling to K_{Ca} channels. *Am J Physiol Cell Physiol* 2000;279:C1938–45.
- [64] Sausbier M, Arntz C, Bucurenciu I, Zhao H, Zhou XB, Sausbier U, Feil S, Kamm S, Essin K, Sailer CA, Abdullah U, Krippeit-Drews P, Feil R, Hofmann F, Knaus HG, Kenyon C, Shipston MJ, Storm JF, Neuhuber W, Korth M, Schubert R, Gollasch M, Ruth P. Elevated blood pressure linked to primary hyperaldosteronism and impaired vasodilation in BK channel-deficient mice. *Circulation* 2005;112:60–8.

- [65] White RE, Lee AB, Shcherbatko AD, Lincoln TM, Schonbrunn A, Armstrong DL. Potassium channel stimulation by natriuretic peptides through cGMP-dependent dephosphorylation. *Nature* 1993;361:263–6.
- [66] Stockand JD, Sansom SC. Mechanism of activation by cGMP-dependent protein kinase of large Ca²⁺-activated K⁺ channels in mesangial cells. *Am J Physiol* 1996;271:C1669–77.
- [67] Han G, Kryman JP, McMillin PJ, White RE, Carrier GO. A novel transduction mechanism mediating dopamine-induced vascular relaxation: opening of BK_{Ca} channels by cyclic AMP-induced stimulation of the cyclic GMP-dependent protein kinase. *J Cardiovasc Pharmacol* 1999;34:619–27.
- [68] White RE, Kryman JP, El-Mowafy AM, Han G, Carrier GO. cAMP-dependent vasodilators cross-activate the cGMP-dependent protein kinase to stimulate BK_{Ca} channel activity in coronary artery smooth muscle cells. *Circ Res* 2000;86:897–905.
- [69] Peng W, Hoidal JR, Farrukh IS. Regulation of Ca²⁺-activated K⁺ channels in pulmonary vascular smooth muscle cells: role of nitric oxide. *J Appl Physiol* 1996;81:1264–72.
- [70] Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 1994;368:850–3.
- [71] Wu L, Cao K, Lu Y, Wang R. Different mechanisms underlying the stimulation of K_{Ca} channels by nitric oxide and carbon monoxide. *J Clin Invest* 2002;110:691–700.
- [72] Barman SA, Zhu S, White RE. PKC activates BK_{Ca} channels in rat pulmonary arterial smooth muscle via cGMP-dependent protein kinase. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L1275–81.
- [73] Barman SA, Zhu S, White RE. Protein kinase C inhibits BK_{Ca} channel activity in pulmonary arterial smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L149–55.
- [74] Zhou XB, Arntz C, Kamm S, Motejlek K, Sausbier U, Wang GX, Ruth P, Korth M. A molecular switch for specific stimulation of the BK_{Ca} channel by cGMP and cAMP kinase. *J Biol Chem* 2001;276:43239–45.
- [75] Zhou XB, Wulfsen I, Utku E, Sausbier U, Sausbier M, Wieland T, Ruth P, Korth M. Dual role of protein kinase C on BK channel regulation. *Proc Natl Acad Sci USA* 2010;107:8005–10.
- [76] Alioua A, Mahajan A, Nishimaru K, Zarei MM, Stefani E, Toro L. Coupling of c-Src to large conductance voltage- and Ca²⁺-activated K⁺ channels as a new mechanism of agonist-induced vasoconstriction. *Proc Natl Acad Sci USA* 2002;99:14560–5.

- [77] Ling S, Woronuk G, Sy L, Lev S, Braun AP. Enhanced activity of a large conductance, calcium-sensitive K^+ channel in the presence of Src tyrosine kinase. *J Biol Chem* 2002;275:30683–9.
- [78] Wolin MS. Reactive oxygen species and the control of vascular function. *Am J Physiol Heart Circ Physiol* 2009;296:H539–49.
- [79] Wang ZW, Nara M, Wang YX, Kotlikoff MI. Redox regulation of large conductance Ca^{2+} -activated K^+ channels in smooth muscle cells. *J Gen Physiol* 1997;110:35–44.
- [80] Zeng XH, Xia XM, Lingle CJ. Redox-sensitive extracellular gates formed by auxiliary β subunits of calcium-activated potassium channels. *Nat Struct Biol* 2003;10:448–54.
- [81] Santarelli LC, Wassef R, Heinemann SH, Hoshi T. Three methionine residues located within the regulator of conductance for K^+ (RCK) domains confer oxidative sensitivity to large-conductance Ca^{2+} -activated K^+ channels. *J Physiol* 2006;571:329–48.
- [82] Miura H, Bosnjak JJ, Ning G, Saito T, Miura M, Gutterman DD. Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. *Circ Res* 2003;92:e31–40.
- [83] Barlow RS, El-Mowafy AM, White RE. H_2O_2 opens BK_{Ca} channels via the PLA_2 -arachidonic acid signaling cascade in coronary artery smooth muscle. *Am J Physiol Heart Circ Physiol* 2000;279:H475–83.
- [84] Kojima K, Kume H, Ito S, Oguma T, Shiraki A, Kondo M, Ito Y, Shimokata K. Direct effects of hydrogen peroxide on airway smooth muscle tone: roles of Ca^{2+} influx and Rho-kinase. *Eur J Pharmacol* 2007;556:151–6.
- [85] Liu Y, Terata K, Chai Q, Li H, Kleinman LH, Gutterman DD. Peroxynitrite inhibits Ca^{2+} -activated K^+ channel activity in smooth muscle of human coronary arterioles. *Circ Res* 2002;91:1070–6.
- [86] Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev* 2002;82:131–85.
- [87] Clarke AL, Petrou S, Walsh JV Jr, Singer JJ. Modulation of BK_{Ca} channel activity by fatty acids: structural requirements and mechanism of action. *Am J Physiol Cell Physiol* 2002;283:C1441–53.
- [88] Morin C, Sirois M, Echave V, Gomes MM, Rousseau E. Functional effects of 20-HETE on human bronchi: hyperpolarization and relaxation due to BK_{Ca} channel activation. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L1037–44.
- [89] Zou AP, Fleming JT, Falck JR, Jacobs ER, Gebremedhin D, Harder DR, Roman RJ. 20-HETE is an endogenous inhibitor of the large-conductance Ca^{2+} -activated K^+ channel in renal arterioles. *Am J Physiol* 1996;270:R228–37.

- [90] Gebremedhin D, Yamaura K, Harder DR. Role of 20-HETE in the hypoxia-induced activation of Ca²⁺-activated K⁺ channel currents in rat cerebral arterial muscle cells. *Am J Physiol Heart Circ Physiol* 2008;294:H107–20.
- [91] Dale PR, Cernecka H, Schmidt M, Dowling MR, Charlton SJ, Pieper MP, Michel MC. The pharmacological rationale for combining muscarinic receptor antagonists and β -adrenoceptor agonists in the treatment of airway and bladder disease. *Curr Opin Pharmacol* 2014;16:31–42.
- [92] Sarria B, Naline E, Zhang Y, Cortijo J, Molimard M, Moreau J, Therond P, Advenier C, Morcillo EJ. Muscarinic M₂ receptors in acetylcholine-isoproterenol functional antagonism in human isolated bronchus. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L1125–32.
- [93] Ikeda T, Anisuzzaman AS, Yoshiki H, Sasaki M, Koshiji T, Uwada J, Nishimune A, Itoh H, Muramatsu I. Regional quantification of muscarinic acetylcholine receptors and β -adrenoceptors in human airways. *Br J Pharmacol* 2012;166:1804–14.
- [94] Rodrigo GJ, Plaza V. Efficacy and safety of a fixed-dose combination of indacaterol and Glycopyrronium for the treatment of COPD: a systematic review. *Chest* 2014;146:309–17.
- [95] Brichetto L, Song P, Crimi E, Rehder K, Brusasco V. Modulation of cholinergic responsiveness through the β -adrenoceptor signal transmission pathway in bovine trachealis. *J Appl Physiol* 2003;95:735–41.
- [96] Giembycz MA, Newton R. Beyond the dogma: novel β_2 -adrenoceptor signalling in the airways. *Eur Respir J* 2006; 27: 1286–306.
- [97] Kume H, Imbe S, Iwanaga T, Tohda Y. Synergistic effects between glycopyrronium bromide and indacaterol on a muscarinic agonist-induced contraction in airway smooth muscle. In: Final Programme of European Respiratory Society Annual Congress (ERS Vienna 2012), 1–5 September 2012; Vienna, Austria, P4845.
- [98] Cazzola M, Calzetta L, Segreti A, Facciolo F, Rogliani P, Matera MG. Translational Study Searching for Synergy between Glycopyrronium and Indacaterol. *COPD* 2014 Sep 15. [Epub ahead of print].
- [99] Kume H, Imbe S, Iwanaga T, Nishiyama O, Higashimoto Y, Tohda Y. Involvement of G proteins in the synergistic effects between anticholinergic agents and β_2 -adrenoceptor agonists in airway smooth muscle. *Am J Respir Crit Care Med* 2013;187:A1996.
- [100] Bateman ED, Mahler DA, Vogelmeier CF, Wedzicha JA, Patalano F, Banerji D. Recent advances in COPD disease management with fixed-dose long-acting combination therapies. *Expert Rev Respir Med* 2014;8:357–79.

- [101] Ozaki H, Kwon SC, Tajimi M, Karaki H. Changes in cytosolic Ca^{2+} and contraction induced by various stimulants and relaxants in canine tracheal smooth muscle. *Pflugers Arch* 1990; 416:351–9.
- [102] Liu B, Freyer AM, Hall IP. Bradykinin activates calcium-dependent potassium channels in cultured human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L898–907.
- [103] Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature* 1994;372:231–6.
- [104] Somlyo AP, Somlyo AV. Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 2003;83:1325–58.
- [105] Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N, Narumiya S. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J* 1996;15:1885–93.
- [106] Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Okawa K, Iwamatsu A, Kaibuchi K. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for the small GTP binding protein Rho. *EMBO J* 1996;15:2208–16.
- [107] Wilson DP, Susnjar M, Kiss E, Sutherland C, Walsh MP. Thromboxane A₂-induced contraction of rat caudal arterial smooth muscle involves activation of Ca^{2+} entry and Ca^{2+} sensitization: rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. *Biochem J* 2005;389:763–74.
- [108] Kume H, Takeda N, Oguma T, Ito S, Kondo M, Ito Y, Shimokata K. Sphingosine 1-phosphate causes airway hyper-reactivity by Rho-mediated myosin phosphatase inactivation. *J Pharmacol Exp Ther* 2007;320:766–73.
- [109] Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, Kaibuchi K. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science* 1997;275:1308–11.
- [110] Hunter I, Cobban HJ, Vandenabeele P, MacEwan DJ, Nixon GF. Tumor necrosis factor- α -induced activation of RhoA in airway smooth muscle cells: role in the Ca^{2+} sensitization of myosin light chain20 phosphorylation. *Mol Pharmacol* 2003;63:714–21.
- [111] Seto M, Sasaki Y, Hidaka H, Sasaki Y. Effects of HA1077, a protein kinase inhibitor, on myosin phosphorylation and tension in smooth muscle. *Eur J Pharmacol* 1991;195:267–72.
- [112] Kobayashi M, Kume H, Oguma T, Makino Y, Ito Y, Shimokata K. Mast cell tryptase causes homologous desensitization of β -adrenoceptors by Ca^{2+} sensitization in tracheal smooth muscle. *Clin Exp Allergy* 2008;38:135–44.

- [113] Oguma T, Ito S, Kondo M, Makino Y, Shimokata K, Honjo H, Kamiya K, Kume H. Roles of P2X receptors and Ca²⁺ sensitization in extracellular adenosine triphosphate-induced hyperresponsiveness in airway smooth muscle. *Clin Exp Allergy* 2007;37: 893–900.
- [114] Shiraki A, Kume H, Oguma T, Makino Y, Ito S, Shimokata K, Honjo H, Kamiya K. Role of Ca²⁺ mobilization and Ca²⁺ sensitization in 8-iso-PGF_{2α}-induced contraction in airway smooth muscle. *Clin Exp Allergy* 2009;39:236–45.
- [115] Bai Y, Sanderson MJ. The contribution of Ca²⁺ signaling and Ca²⁺ sensitivity to the regulation of airway smooth muscle contraction is different in rats and mice. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L947–58.
- [116] Lan B, Deng L, Donovan GM, Chin LY, Syyong HT, Wang L, Zhang J, Pascoe CD, Norris BA, Liu JC, Swynghedouw NE, Banaem SM, Paré PD, Seow CY. Force maintenance and myosin filament assembly regulated by Rho-kinase in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2015;308:L1–10.
- [117] Bai TR. Abnormalities in airway smooth muscle in fatal asthma. *Am Rev Respir Dis* 1990;141: 552–7.
- [118] Schmidt D, Rabe KF. Immune mechanisms of smooth muscle hyperreactivity in asthma. *J Allergy Clin Immunol* 2000;105:673–82.
- [119] Rizzo CA, Yang R, Greenfeder S, Egan RW, Pauwels RA, Hey JA. The IL-5 receptor on human bronchus selectively primes for hyperresponsiveness. *J Allergy Clin Immunol* 2002;109:404–9.
- [120] Tliba O, Deshpande D, Chen H, Van Besien C, Kannan M, Panettieri RA Jr, Amrani Y. IL-13 enhances agonist-evoked calcium signals and contractile responses in airway smooth muscle. *Br J Pharmacol* 2003;140:1159–62.
- [121] Kudo M, Melton AC, Chen C, Engler MB, Huang KE, Ren X, Wang Y, Bernstein X, Li JT, Atabai K, Huang X, Sheppard D. IL-17A produced by αβ T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. *Nat Med* 2012;18:547–54.
- [122] Setoguchi H, Nishimura J, Hirano K, Takahashi S, Kanaide H. Leukotriene C₄ enhances the contraction of porcine tracheal smooth muscle through the activation of Y-27632, a rho kinase inhibitor, sensitive pathway. *Br J Pharmacol* 2001;132:111–8.
- [123] Rosenfeldt HM, Amrani Y, Watterson KR, Murthy KS, Panettieri RA Jr, Spiegel S. Sphingosine-1-phosphate stimulates contraction of human airway smooth muscle cells. *FASEB J* 2003;17:1789–99.
- [124] Liu C, Tazzeo T, Janssen LJ. Isoprostane-induced airway hyperresponsiveness is dependent on internal Ca²⁺ handling and Rho/ROCK signaling. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L1177–84.

- [125] Chiba Y, Sato S, Hanazaki M, Sakai H, Misawa M. Inhibition of geranylgeranyltransferase inhibits bronchial smooth muscle hyperresponsiveness in mice. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L984–91.
- [126] Gosens R, Schaafsma D, Meurs H, Zaagsma J, Nelemans SA. Role of Rho-kinase in maintaining airway smooth muscle contractile phenotype. *Eur J Pharmacol* 2004;483:71–8.
- [127] An SS, Fabry B, Treppe X, Wang N, Fredberg JJ. Do biophysical properties of the airway smooth muscle in culture predict airway hyperresponsiveness? *Am J Respir Cell Mol Biol* 2006;35:55–64.
- [128] Taki F, Kume H, Kobayashi T, Ohta H, Aratake H, Shimokata K. Effects of Rho-kinase inactivation on eosinophilia and hyper-reactivity in murine airways by allergen challenges. *Clin Exp Allergy* 2007;37:599–607.
- [129] Tao FC, Tolloczko B, Eidelman DH, Martin JG. Enhanced Ca^{2+} mobilization in airway smooth muscle contributes to airway hyperresponsiveness in an inbred strain of rat. *Am J Respir Crit Care Med* 1999;160:446–53.
- [130] Cheng YM, Cao AL, Zheng JP, Wang HW, Sun YS, Liu CF, Zhang BB, Wang Y, Zhu SL, Wu DZ. Airway hyperresponsiveness induced by repeated esophageal infusion of HCl in guinea pigs. *Am J Respir Cell Mol Biol* 2014;51:701–8.
- [131] Benovic JL, Strasser RH, Caron MG, Lefkowitz RJ. β -adrenergic receptor kinase: identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. *Proc Natl Acad Sci USA* 1986;83:2797–801.
- [132] Clark RB, Kunkel MW, Friedman J, Goka TJ, Johnson JA. Activation of cAMP-dependent protein kinase is required for heterologous desensitization of adenylyl cyclase in S49 wild-type lymphoma cells. *Proc Natl Acad Sci USA* 1988;85:1442–6.
- [133] Hausdorff WP, Bouvier M, O'Dowd BF, Irons GP, Caron MG, Lefkowitz RJ. Phosphorylation sites on two domains of the β_2 -adrenergic receptor are involved in distinct pathways of receptor desensitization. *J Biol Chem* 1989;264:12657–65.
- [134] Kume H, Takagi K. Inhibitory effects of Gs on desensitization of β -adrenergic receptors in tracheal smooth muscle. *Am J Physiol* 1997;273:L556–64.
- [135] Kume H, Takagi K. Inhibition of β -adrenergic desensitization by K_{Ca} channels in human trachealis. *Am J Respir Crit Care Med* 1999;159:452–60.
- [136] Mizutani H, Kume H, Ito Y, Takagi K, Yamaki K. Different effects of β -adrenoceptor desensitization on inhibitory actions in guinea-pig trachealis. *Clin Exp Pharmacol Physiol* 2002;29:646–54.
- [137] Koto H, Mak JC, Haddad EB, Xu WB, Salmon M, Barnes PJ, Chung KF. Mechanisms of impaired β -adrenoceptor-induced airway relaxation by interleukin-1 β in vivo in the rat. *J Clin Invest* 1996;98:1780–7.

- [138] Ishikawa T, Kume H, Kondo M, Ito Y, Yamaki K, Shimokata K. Inhibitory effects of interferon- γ on the heterologous desensitization of β -adrenoceptors by transforming growth factor- β 1 in tracheal smooth muscle. *Clin Exp Allergy* 2003;33:808–15.
- [139] Ikenouchi T, Kume H, Oguma T, Makino Y, Shiraki A, Ito Y, Shimokata K. Role of Ca²⁺ mobilization in desensitization of β -adrenoceptors by platelet-derived growth factor in airway smooth muscle. *Eur J Pharmacol* 2008;591:259–65.
- [140] Kume H, Ito S, Ito Y, Yamaki K. Role of lysophosphatidylcholine in the desensitization of β -adrenergic receptors by Ca²⁺ sensitization in tracheal smooth muscle. *Am J Respir Cell Mol Biol* 2001;25:291–8.
- [141] Makino Y, Kume H, Oguma T, Sugishita M, Shiraki A, Hasegawa Y, Honjo H, Kamiya K. Role of sphingosine-1-phosphate in β -adrenoceptor desensitization via Ca²⁺ sensitization in airway smooth muscle. *Allergol Int* 2012;61:311–22.
- [142] Sudo Y, Kume H, Ito S, Ito Y, Yamaki K, Takagi K. Effects of direct and indirect activation of G protein of adenylyl cyclase on the subsequent response to β -adrenergic receptor agonists in human trachealis. *Arzneimittelforschung* 2002;52:803–12.
- [143] Finney PA, Belvisi MG, Donnelly LE, Chuang TT, Mak JC, Scorer C, Barnes PJ, Adcock IM, Giembycz MA. Albuterol-induced downregulation of Gs α accounts for pulmonary β_2 -adrenoceptor desensitization in vivo. *J Clin Invest* 2000;106:125–35.
- [144] Hanania NA, Sharafkhaneh A, Barber R, Dickey BF. β -agonist intrinsic efficacy: measurement and clinical significance. *Am J Respir Crit Care Med* 2002;165:1353–8.
- [145] Kume H. Clinical Use of β_2 -adrenergic receptor agonists based on their intrinsic efficacy. *Allergol Int* 2005;54:89–97.
- [146] Kume H, Kondo M, Ito Y, Suzuki R, Yamaki K, Takagi K. Effects of sustained-release tulobuterol on asthma control and β -adrenoceptor function. *Clin Exp Pharmacol Physiol* 2002;29:1076–83.
- [147] Bara I, Ozier A, Tunon de Lara JM, Marthan R, Berger P. Pathophysiology of bronchial smooth muscle remodelling in asthma. *Eur Respir J* 2010;36:1174–84.
- [148] Girodet PO, Ozier A, Bara I, Tunon de Lara JM, Marthan R, Berger P. Airway remodeling in asthma: new mechanisms and potential for pharmacological intervention. *Pharmacol Ther* 2011;130:325–37.
- [149] Roth M, Johnson PR, Borger P, Bihl MP, Rüdiger JJ, King GG, Ge Q, Hostettler K, Burgess JK, Black JL, Tamm M. Dysfunctional interaction of C/EBP α and the glucocorticoid receptor in asthmatic bronchial smooth-muscle cells. *N Engl J Med* 2004;351:560–74.
- [150] Hirst SJ, Martin JG, Bonacci JV, Chan V, Fixman ED, Hamid QA, Herszberg B, Lavoie JP, McVicker CG, Moir LM, Nguyen TT, Peng Q, Ramos-Barbón D, Stewart AG. Proliferative aspects of airway smooth muscle. *J Allergy Clin Immunol* 2004;114:S2–17.

- [151] Billington CK, Kong KC, Bhattacharyya R, Wedegaertner PB, Panettieri RA Jr, Chan TO, Penn RB. Cooperative regulation of p70S6 kinase by receptor tyrosine kinases and G protein-coupled receptors augments airway smooth muscle growth. *Biochemistry* 2005;44:14595–605.
- [152] Gosens R, Dueck G, Rector E, Nunes RO, Gerthoffer WT, Unruh H, Zaagsma J, Meurs H, Halayko AJ. Cooperative regulation of GSK-3 by muscarinic and PDGF receptors is associated with airway myocyte proliferation. *Am J Physiol Lung Cell Mol Physiol* 2007;293: L1348–58.
- [153] Takeda N, Kondo M, Ito S, Ito Y, Shimokata K, Kume H. Role of RhoA inactivation in reduced cell proliferation of human airway smooth muscle by simvastatin. *Am J Respir Cell Mol Biol* 2006 ;35:722–9.
- [154] Oenema TA, Mensink G, Smedinga L, Halayko AJ, Zaagsma J, Meurs H, Gosens R, Dekkers BG. Cross-talk between transforming growth factor- β_1 and muscarinic M₂ receptors augments airway smooth muscle proliferation. *Am J Respir Cell Mol Biol*. 2013;49:18–27.
- [155] Placeres-Uray FA, Febres-Aldana CA, Fernandez-Ruiz R, Gonzalez de Alfonso R, Lippo de Becemberg IA, Alfonso MJ. M₂ Muscarinic acetylcholine receptor modulates rat airway smooth muscle cell proliferation. *World Allergy Organ J* 2013;6: 22.
- [156] Girodet PO, Dournes G, Thumerel M, Begueret H, Dos Santos P, Ozier A, Dupin I, Trian T, Montaudon M, Laurent F, Marthan R, Berger P. Calcium Channel Blocker Reduces Airway Remodeling in Severe Asthma: a Proof-of-concept Study. *Am J Respir Crit Care Med* 2015;191:876–83.
- [157] Madison JM. Migration of airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2003;29:8–11.
- [158] Parameswaran K, Radford K, Zuo J, Janssen LJ, O'Byrne PM, Cox PG. Extracellular matrix regulates human airway smooth muscle cell migration. *Eur Respir J* 2004;24:545–51.
- [159] Hirshman CA, Emala CW. Actin reorganization in airway smooth muscle cells involves G_q and G_{i-2} activation of Rho. *Am J Physiol* 1999;277:L653–61.
- [160] Irani C, Goncharova EA, Hunter DS, Walker CL, Panettieri RA, Krymskaya VP. Phosphatidylinositol 3-kinase but not tuberlin is required for PDGF-induced cell migration. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L854–62.
- [161] Parameswaran K, Cox G, Radford K, Janssen LJ, Sehmi R, O'Byrne PM. Cysteinyl leukotrienes promote human airway smooth muscle migration. *Am J Respir Crit Care Med* 2002;166:738–42.
- [162] Carlin SM, Resink TJ, Tamm M, Roth M. Urokinase signal transduction and its role in cell migration. *FASEB J* 2005;19:195–202.

- [163] Hirakawa M, Karashima Y, Watanabe M, Kimura C, Ito Y, Oike M. Protein kinase A inhibits lysophosphatidic acid-induced migration of airway smooth muscle cells. *J Pharmacol Exp Ther* 2007;321:1102–8.
- [164] Hedges JC, Dechert MA, Yamboliev IA, Martin JL, Hickey E, Weber LA, Gerthoffer WT. A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. *J Biol Chem* 1999;274:24211–9.
- [165] Parameswaran K, Radford K, Fanat A, Stephen J, Bonnans C, Levy BD, Janssen LJ, Cox PG. Modulation of human airway smooth muscle migration by lipid mediators and Th-2 cytokines. *Am J Respir Cell Mol Biol* 2007;37:240–7.
- [166] Suganuma N, Ito S, Aso H, Kondo M, Sato M, Sokabe M, Hasegawa Y. STIM1 regulates platelet-derived growth factor-induced migration and Ca²⁺ influx in human airway smooth muscle cells. *PLoS One* 2012;7:e45056.
- [167] Li M, Shang YX, Wei B, Yang YG. The effect of substance P on asthmatic rat airway smooth muscle cell proliferation, migration, and cytoplasmic calcium concentration in vitro. *J Inflamm (Lond)* 2011;8:18, doi: 10.1186/1476-9255-8-18.
- [168] Matsumoto H, Hirata Y, Otsuka K, Iwata T, Inazumi A, Niimi A, Ito I, Ogawa E, Muro S, Sakai H, Chin K, Oku Y, Mishima M. Interleukin-13 enhanced Ca²⁺ oscillations in airway smooth muscle cells. *Cytokine* 2012;57:19–24.
- [169] Nishiyama O, Kume H, Kondo M, Ito Y, Ito M, Yamaki K. Role of lysophosphatidylcholine in eosinophil infiltration and resistance in airways. *Clin Exp Pharmacol Physiol* 2004;31:179–84.
- [170] Fuerst E, Foster HR, Ward JP, Corrigan CJ, Cousins DJ, Woszczek G. Sphingosine-1-phosphate induces pro-remodelling response in airway smooth muscle cells. *Allergy* 2014;69:1531–9.
- [171] Sashio T, Kume H, Takeda N, Asano T, Tsuji S, Kondo M, Hasegawa Y, Shimokata K. Possible involvement of sphingosine-1-phosphate/Gi/RhoA pathways in adherence of eosinophils to pulmonary endothelium. *Allergol Int* 2012;61:283–93.
- [172] Schaafsma D, Bos IS, Zuidhof AB, Zaagsma J, Meurs H. The inhaled Rho kinase inhibitor Y-27632 protects against allergen-induced acute bronchoconstriction, airway hyperresponsiveness, and inflammation. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L214–9.
- [173] Schaafsma D, Gosens R, Zaagsma J, Halayko AJ, Meurs H. Rho-kinase inhibitors: a novel therapeutical intervention in asthma? *Eur J Pharmacol* 2008;585:398–406.
- [174] Possa SS, Charafeddine HT, Righetti RF, da Silva PA, Almeida-Reis R, Saraiva-Romanholo BM, Perini A, Prado CM, Leick-Maldonado EA, Martins MA, Tibério Ide F. Rho-kinase inhibition attenuates airway responsiveness, inflammation, matrix remodeling, and oxidative stress activation induced by chronic inflammation. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L939–52.

- [175] Gerthoffer WT, Solway J, Camoretti-Mercado B. Emerging targets for novel therapy of asthma. *Curr Opin Pharmacol* 2013;13:324–30.
- [176] Perusquía M, Flores-Soto E, Sommer B, Campuzano-González E, Martínez-Villa I, Martínez-Banderas AI, Montaña LM. Testosterone-induced relaxation involves L-type and store-operated Ca^{2+} channels blockade, and PGE_2 in guinea pig airway smooth muscle. *Pflugers Arch* 2015;467:767–77.
- [177] Deshpande DA, Wang WC, McIlmoyle EL, Robinett KS, Schillinger RM, An SS, Sham JS, Liggett SB. Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction. *Nat Med* 2010;16:1299–304.