[Frontiers in Bioscience 15, 750-764, January 1, 2010]

Mechanisms of airway smooth muscle relaxation induced by beta2-adrenergic agonists

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1. ABSTRACT

Airway smooth muscle cell (ASMC) contraction is regulated by myosin phosphorylation to control actin-myosin cross-bridge activity. Myosin phosphorylation is determined by the antagonistic activity of myosin light chain (MLC) kinase (MLCK) and phosphatase (MLCP). MLCK activity is increased by increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) associated with Ca^{2+} oscillations. MLCP activity is decreased by phosphorylation of MLCP or accessory proteins by kinases, including Rho-kinase or protein kinase C. During agonist-induced ASMC contraction, these 2 pathways are simultaneously activated. Because MLCP activity is often independent of $[Ca^{2+}]_i$, changes in MLCP activity can alter ASMC tone at a constant $[Ca^{2+}]_i$; a behavior termed Ca^{2+} sensitivity. In asthma, airway hyperresponsiveness (AHR) may result from an increase in the Ca^{2+}-dependent contractile mechanisms and/or the Ca^{2+} sensitivity of ASMCs. Conversely, inhalation of beta2-adrenergic agonists induce airway relaxation by simultaneously slowing the Ca^{2+} oscillations and reducing the Ca^{2+} sensitivity of ASMCs. However, the action of beta2-adrenergic agonists varies with species. Consequently, the development of beta2-adrenergic agonists requires a characterization of their action in human airways.

2. INTRODUCTION

Airway hyperresponsiveness (AHR) is characteristic of asthma but the conditions that predispose individuals to the development of this disease remain uncertain. However, it is clear that acute episodes of AHR result from excessive or prolonged contraction of airway smooth muscle cells (ASMCs). The force produced by the ASMCs determines the extent of airway contraction and this is a function of their number and size, in balance with the resistive load of the airway wall. As a result, an abnormal increase in force production by individual ASMCs could account for AHR. The recurrent airway inflammation associated with asthma may contribute to AHR by promoting ASMC proliferation and airway wall remodeling. Irrespective of the cause or stimulus evoking an asthma attack, the predominant rescue therapy is the inhalation of beta2-adrenergic agonists that act by relaxing the ASMCs. Long-term control of asthma is aimed at avoiding acute episodes of AHR and involves the suppression of inflammation with steroids or cytokine receptor antagonists.

3. BETA2-ADRENERGIC AGONISTS

Because beta2-adrenergic agonists have been in prominent use as an asthma therapy for several decades, it

is surprising that their mechanism of action at the cellular level is poorly characterized. This lack of an understanding has been, perhaps, acceptable in view of the efficacy of these beta2-agonists. A short-acting beta2-adrenergic agonist (SABA) commonly used as a rescue therapy for asthma is albuterol. In moderate to severe asthma or chronic pulmonary disease, where greater therapeutic control is required, a combination of inhaled glucocorticoid steroids (fluticasone or budesonide) with a long-acting beta2-adrenergic agonist (LABA) such as formoterol is increasingly being used (1). While, albuterol and formoterol appear to act directly on ASMCs, formoterol may also act indirectly by inhibiting mast cells from releasing or producing contractile stimuli or by enhancing the ability of glucocorticoid steroids to down-regulate inflammation (1). A beneficial characteristic of LABAs is their ability to sustain airway relaxation for many hours (8 – 12 hours) as compared to SABAs (4 hours). This property is believed to be the result of the lipophilic structure of LABAs which would allow the beta2-agonist to more easily partition into the ASMC membrane where it may have access to an internal regulation site of the beta2-adrenergic receptor. Such an internal activation may avoid receptor de-sensitization via phosphorylation by protein kinase A (PKA), beta-adrenergic receptor kinase and G-coupled receptor kinase or the binding of beta-arrestin. However, it is unclear if an internal activation of the beta2-adrenergic receptor induces ASMC relaxation in a manner identical to that of external activation by SABAs. Alternatively, the sequestration of lipophilic LABAs within cell membranes may form a slow-release reservoir of beta2-agonist that could continually stimulate the ASMCs (2).

Despite the advantages of beta2-adrenergic agonists, the efficacy of albuterol has been reported to be reduced with continual and frequent use and may even exacerbate bronchial constriction (3, 4). One explanation for this effect could be the desensitization of the beta2-adrenergic receptor (5). In mouse lung slices, ASMCs quickly de-sensitize (upon a single exposure) to isoproterenol (5). Surprisingly, repetitive exposures to equal concentrations of albuterol or formoterol induced little tachyphylaxis (6). This suggests that albuterol and formoterol may stimulate the beta-adrenergic receptor in a different way to that of isoproterenol and that this avoids the receptor-desensitization mechanisms. Although clinical studies with salmeterol implied substantial receptor desensitization to recommend against its use alone (7), the desensitization responses to formoterol are not well understood in human airways. However, prolonged exposure to albuterol desensitized the beta-receptor response to isoproterenol in human lung slices (8). While this experimental result correlated with a loss of beta2-receptors, it was not clear if the clinical parallel of albuterol-induced desensitization to subsequent use of albuterol occurred. Our data (6) suggest that albuterol and formoterol do not fully mimic isoproterenol with respect to the mechanism by which they exert their relaxation response, and this difference also appears to apply to the desensitization of the receptor by albuterol. Although the implications of albuterol-induced receptor desensitization to isoproterenol are not clear, the major clinical approach to address receptor desensitization is the reduced usage of beta2-agonists. The alternative clinical approach of the combined use of beta2-agonists with steroids, matches the finding that steroids reversed albuterol-induced desensitization of the beta2-receptor to isoproterenol (8).

Another mechanism proposed for reduced responsiveness to beta2-agonists is the presence of agonist enantiomers in clinical formulations; albuterol usually contains a racemic mix of approximately 50% of (R)- and (S)-albuterol. Similarly, formoterol contains a mix of 50% (R,R)- and (S,S)-formoterol. (R)-albuterol, in comparison to (S)-albuterol, strongly binds to beta2-adrenergic receptors and is metabolized quickly; as a result (S)-albuterol may accumulate within the lungs. (R,R)-formoterol binds with a 1000-fold greater affinity for the human beta2-adrenergic receptor and has a 640-fold greater potency to relax isolated guinea-pig tracheal SMC strips (contracted with histamine) than (S,S)-formoterol (9).

While these pharmacological differences are consistent with the idea that S-enantiomers have little effect, clinical trials reported an improved FEV_1 in patients treated with only (R)-albuterol as compared to racemic albuterol; the implication being that elevated levels of (S)-albuterol hinder (R)-albuterol-induced airway relaxation (10). Henderson *et al.*, (11) also reported that (S)-albuterol increased allergen-induced edema and AHR in a mouse asthma model. The relaxant effects of (R,R)-formoterol also appeared greater than those of racemic formoterol (9), and when (S,S)-formoterol was used in conjunction with the contractile agonist carbachol, it appeared to enhance airway contraction. These contractile actions of (S)-albuterol or (S,S)-formoterol may be indirect as both enantiomers have been reported to increase histamine and cytokine (IL-4) production by mast cells (12, 13). On the other hand, several studies have shown no significant differences between the action of (R)-albuterol and racemic albuterol (14, 15).

Understanding how S-enantiomers may antagonize relaxation induced by R-enantiomers or induce contraction themselves is an important issue for beta2-adrenergic agonist based therapy, but only a few studies have addressed this issue at the ASMC level (16-18). These studies indicated that (S)-albuterol increased $[Ca^{2+}]_i$, but the use of isolated or cultured ASMCs limited the ability to correlate the dynamics of ASMC contraction or relaxation with cell signaling in response to agonists. More importantly, the mechanisms mediating ASMC contraction are not fully understood and this has the obvious consequence that the specifics by which beta2-adrenergic agonists induce airway relaxation remain vague. Consequently, we initially review here, the basic mechanisms of ASMC contraction which serves as the prerequisite for our discussion of how these mechanisms are influenced by beta2-adrenergic agonists to induce airway relaxation.

4. FUNDAMENTALS OF AIRWAY SMOOTH MUSCLE CONTRACTION

The contractile machinery of ASMCs consists of overlapping actin and myosin filaments that interact to

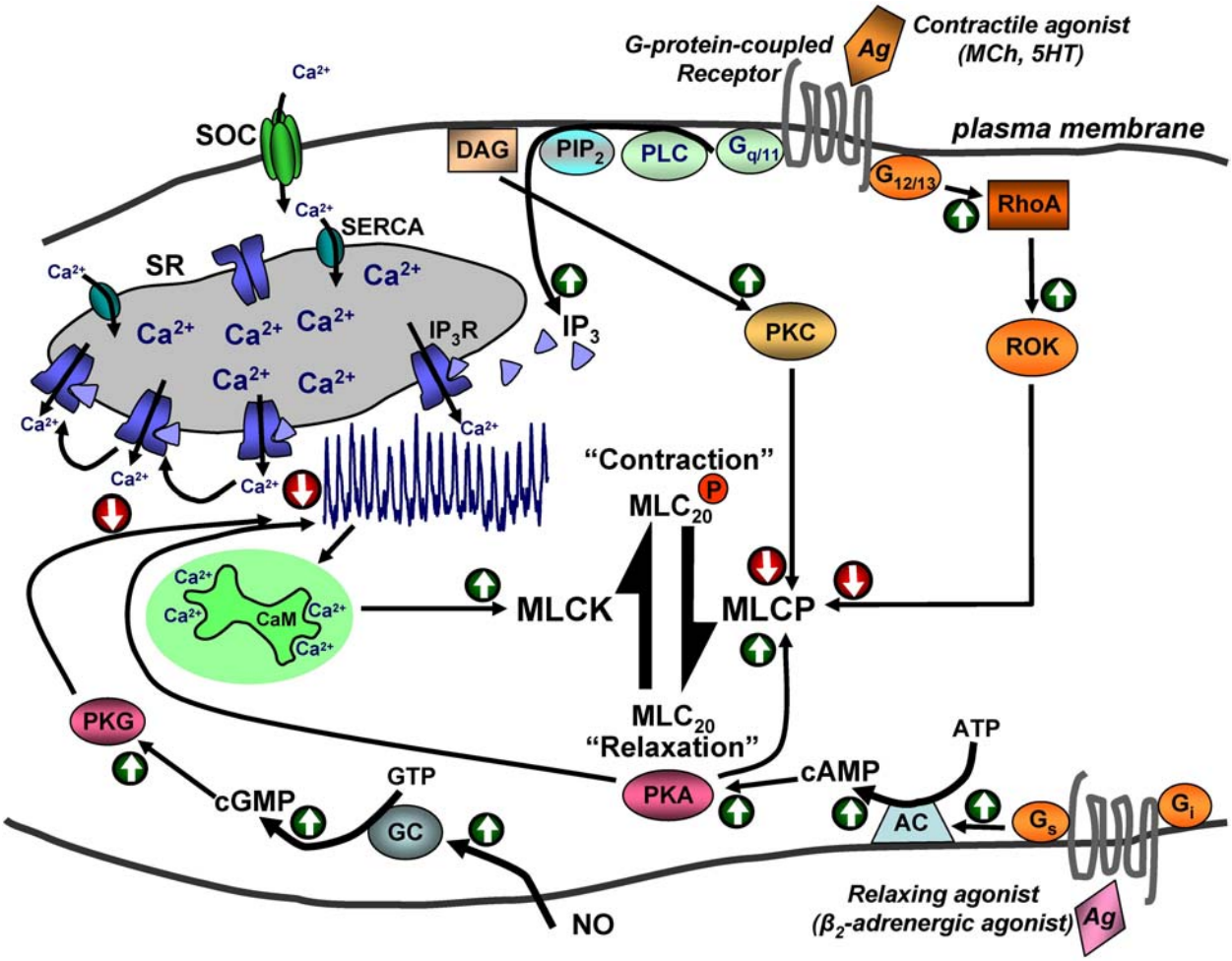

Figure 1. Signal transduction pathways mediating ASMC contraction and relaxation. Binding of a contractile agonist (e.g. MCh) to its receptor (mainly G-protein-coupled receptors) activates (up arrow/green) phospholipase C (PLC) to produce inositol trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidyl inositol bisphosphate (PIP_2). IP_3 binds to receptors (IP_3R) on the sarcoplasmic reticulum (SR), thereby opening the receptor and initiating the release of Ca^{2+} from the SR into the cytoplasm. The release of Ca^{2+} occurs in an oscillatory manner and propagates along the cell in the form of a Ca^{2+} wave. The Ca^{2+} binds to calmodulin (CaM) and this complex activates myosin light chain kinase (MLCK). Activated MLCK phosphorylates the 20 kDa light chain of myosin (MLC_{20}) which initiates the formation of actin-myosin cross-bridges that lead to contraction. DAG activates protein kinase C (PKC), which may lead to the inhibition (down arrow/red) of myosin light chain phosphatase (MLCP) and the enhancement of contraction by decreasing the de-phosphorylation of MLC_{20}. Activation of the G-protein-coupled receptor simultaneously leads to the activation of RhoA and Rho kinase (ROK) which also leads to the subsequent inhibition of MLCP. The binding of a relaxant beta2-adrenergic agonist to the G-protein coupled beta2-adrenergic receptor leads to production of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) via the activation of adenylate cyclase (AC). Increases in cAMP, presumably acting via protein kinase A (PKA), slow or abolish Ca^{2+} oscillations, either by inhibition of the IP_3R or a decrease in $[IP_3]$, resulting from a decrease in PLC activity. In addition, cAMP/PKA also decreases Ca^{2+} sensitivity which implies an activation of MLCP to de-phosphorylate MLC_{20}. Nitric oxide (NO) activates guanylate cyclase (GC) to stimulate cyclic guanosine monophosphate (cGMP) production, activation of protein kinase G (PKG) and a decrease in the Ca^{2+} oscillation frequency.

generate force by filament sliding. The regulation of this contractile behavior is dependent of on the phosphorylation state of the myosin filament; when myosin (i.e. the myosin light chain (MLC)) is phosphorylated, it forms a cross-bridge with the actin filament and undergoes cyclic molecular transformations, utilizing ATP, to generate sliding (19). However, some filament assembly, that serve as membrane anchoring sites to couple the internal sliding forces to the external environment, is also initially required (20). Phosphorylation of myosin also stabilizes the myosin filaments during contraction (21).

The balance of the antagonistic activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) (Figure 1) primarily determines the phosphorylation of the MLC. MLCK activity is, for the

most part, regulated by calmodulin and is a function of the $[Ca^{2+}]_i$. On the other hand, MLCP activity is commonly independent of $[Ca^{2+}]_i$ (except in mouse ASMCs (22)) and regulated by phosphorylation of associated regulatory proteins through the activities of a variety of other kinases or phosphatases (Figure 1). This molecular design provides considerable versatility and has interesting implications for the regulation of force production; increases in MLCK activity and decreases in MLCP activity, either simultaneously or independently, will result in increased force production. Conversely, decreases in MLCK activity or increases in MLCP activity will induce ASMC relaxation. An important implication of this molecular design is that the contractile state of the ASMC can be altered by mechanisms other than changes in $[Ca^{2+}]_i$ and MLCK activity; a behavior traditionally referred to as "Ca^{2+} sensitivity". While MLCP activity probably accounts for most of the changes in contractility associated with Ca^{2+} sensitivity, events that do not alter MLC phosphorylation, (i.e. the binding of the accessory protein caldesmon to alter actin-myosin interactions) may also contribute to Ca^{2+} sensitivity.

A second consequence of the antagonistic action of MLCK and MLCP is the generation of a "latch" bridge – an un-phosphorylated myosin cross-bridge that does not actively generate force but remains attached to actin because of its slow dissociation rate. This cross-bridge configuration resists filament sliding and, if an excess of latch-bridges are formed, they will prevent the ASMCs from relaxing. Therefore, the observed increases in ASMC tone associated with asthma or COPD may result from the combined alterations of MLCK and MLCP activity in response to a variety of putative or inflammatory signaling molecules. Effective therapeutic intervention requires an understanding of the relative contribution and mechanisms of each process.

4.1. Airway physiology revealed with lung slices

In an effort to address the mechanisms of contraction and relaxation, we have conducted studies of agonist-induced airway contraction (Figure 2) as well as relaxation (Figures 3 and 4) using lung slices (5, 23-27). The advantages of this preparation include the retention of the *in situ* features of airways, such as a ciliated epithelium, intact ASMCs and airway tethering to the surrounding alveolar tissue. The ASMCs remain active and can undergo multiple cycles of contraction and relaxation (Figure 2). In addition, lung slices are viable for many days and can be obtained from mouse, rat (28), guinea pig (29) and humans (8, 27, 29-31). Importantly, lung slices are highly compatible with microscopy techniques and we have developed approaches that allow a quantitative and correlative analysis of the simultaneous changes in airway contractility with the $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of individual ASMCs with mouse, rat and human lung slices. This information has been vital for guiding subsequent studies to identify which contractile mechanisms are modified by beta2-adrenergic agonists to induce relaxation (5, 22) and how this activity was compromised by S-enantiomers (6, 32) as well as the validity of extrapolating results from rodent to human airways.

5. CALCIUM SIGNALING IN AIRWAY SMOOTH MUCLE CELLS

With advances in microscopy and single cell observations, contractile agonist-induced Ca^{2+} increases have been commonly observed to occur as repetitive Ca^{2+} oscillations in ASMCs of many species, including mouse (24, 25), pig (33) and human (27, 34). After an initial increase in $[Ca^{2+}]_i$, the Ca^{2+} oscillations stabilize with a steady frequency (Figure 2). Each Ca^{2+} oscillation usually occurs as a Ca^{2+} wave that is initiated at one end of the cell and propagates along the SMCs (Figure 2). Importantly, the frequency of the Ca^{2+} oscillations increases with the contractile agonist concentration and an increased frequency correlates with an increased airway contraction. However, the amplitude of the Ca^{2+} oscillations remains similar, although the base line of the Ca^{2+} oscillation is often elevated (Figure 2). This behavior is consistent with the hypothesis that the frequency of the Ca^{2+} oscillations regulates ASMC contraction. Mathematical modeling suggests that Ca^{2+} oscillations are more effective than steady-state $[Ca^{2+}]_i$ for force generation, but the same model also predicts that the variation of the frequency component of the Ca^{2+} oscillations alone is not responsible for this effect (35). The duration of the Ca^{2+} elevation associated with each Ca^{2+} oscillation also varies with frequency and it is likely that an associated change in average $[Ca^{2+}]_i$ has an influence on contraction (35, 36).

However, there are major differences between the relationships of Ca^{2+} oscillation frequency and airway contraction for different contractile agonists and for airways from different species. For example, MCh (1 microM) induced Ca^{2+} oscillations with a higher frequency (20/min) in mouse ASMCs as compared to rat ASMCs (6/min) but the rat airway displayed a greater contraction (28). Human airways, like rat airways, show slow frequency Ca^{2+} oscillations in response to MCh. The explanation for these different frequency-contraction relationships appears to reside with a greater Ca^{2+} sensitivity of rat and human ASMCs as compared to mouse ASMCs. In Ca^{2+}-permeabilized ASMCs, in which the $[Ca^{2+}]_i$ is maintained at a high steady level, mouse airways are almost fully relaxed (low Ca^{2+} sensitivity) (22) whereas rat (28) and human airways remain contracted (Figure 5). It is interesting to speculate that a higher Ca^{2+} oscillation frequency might compensate for a low Ca^{2+} sensitivity and that slower Ca^{2+} oscillations in rats may reflect a higher Ca^{2+} sensitivity. These different relationships highlight the importance of the relative contribution of the frequency of Ca^{2+} oscillations and Ca^{2+} sensitivity to ASMC contraction and underscore the need for caution when extrapolating airway behavior from experimental animals to humans.

5.1. Mechanisms of agonist-induced calcium oscillations

In the absence of extracellular Ca^{2+}, contractile agonists can initiate, but not sustain, contraction and Ca^{2+} oscillations in ASMCs. This response indicates that Ca^{2+} oscillations result from the repetitive release of Ca^{2+} from internal stores that require supplemental Ca^{2+} influx. The inability of voltage-gated Ca^{2+} channel blockers to inhibit agonist-induced contraction is consistent with the

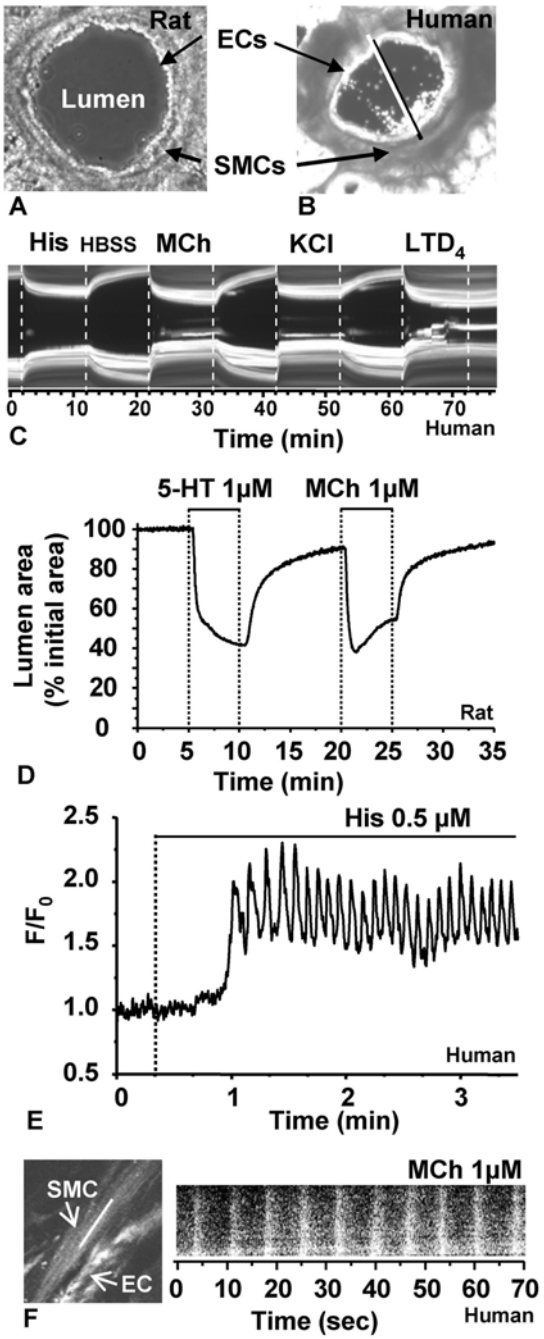

Figure 2. Correlation of Ca^{2+}-signaling and airway contraction in lung slices from different species. (A and B) Phase-contrast images of an airway in rat (A) and human (B) lung slices. Epithelial cells (ECs), airway smooth muscle cells (SMCs), white line = scan line. (C) A line-scan analysis of the human lung slice along the line drawn in B (scan line) showing the contractile response of the airway in response to histamine (His), methacholine (MCh), potassium (KCl) and leukotriene D4 (LTD_4) with respect to time. The airway relaxed in response to perfusion with saline (HBSS) after the exposure to each contractile agonist. (D) The contractile response of a rat airway, as measured by a change in lumen area, to serotonin (5-HT, 1 microM) and MCh (1 microM) with respect to time. (E) Representative Ca^{2+} oscillations, recorded with two-photon laser scanning microscopy, that occur in human ASMCs in a lung slice in response to a contractile agonist (0.5 microM histamine). The change in fluorescence intensity (F) representing increases in $[Ca^{2+}]_i$ is plotted relative (F/F_0) to the initial intensity (F_0) with respect to time. (F) A line-scan analysis with respect to time of the Ca^{2+} changes in a human ASMC (along white-line, left image) in response to MCh. The sloping white bars represent the Ca^{2+} waves as they propagate along the length of the cell. The slope of the line represents the wave propagation velocity. The wave direction was from bottom to top.

dependence of contraction on internal Ca^{2+} release. Although, the details of the internal Ca^{2+} release mechanism in ASMCs may vary between different species or from different locations within the airway, the basic process involves Ca^{2+}-induced Ca^{2+} release (CICR) via the inositol 1,4,5 trisphosphate (IP_3) receptor (IP_3R) of the sarcoplasmic reticulum (SR) (19, 28). An alternative mechanism proposes the involvement of the ryanodine receptor (RyR) (37).

5.1.1. Contribution of inositol trisphosphate receptors

A commonly accepted mechanism for agonist-induced Ca^{2+} oscillations is the activation of the IP_3R by the binding of IP_3 (38, 39) (Figure 1). IP_3, produced by membrane phospholipase C (PLC) via the activation of a G-protein coupled receptor, binds to, and opens the IP_3R to allow Ca^{2+} efflux from the SR. The local increase in $[Ca^{2+}]_i$ favors the binding of a Ca^{2+} ion to the IP_3R which enhances the IP_3R open probability. As a result, $[Ca^{2+}]_i$ increases further and Ca^{2+} diffusion to neighboring sensitized IP_3Rs stimulates additional Ca^{2+} release which leads to the propagation of a Ca^{2+} wave along the airway SMC. At higher $[Ca^{2+}]_i$, the binding of a second Ca^{2+} to the IP_3R reduces its open probability and this, together with the re-accumulation of Ca^{2+} into the SR, decreases in $[Ca^{2+}]_i$ (Figure 1). Indirect evidence for IP_3-based Ca^{2+} oscillations in tracheal SMCs was initially provided by the inhibition of acetylcholine (ACh)-induced Ca^{2+} oscillations with IP_3R antibodies or the IP_3R antagonist, heparin (40). Direct evidence for IP_3-based Ca^{2+} oscillations comes from the stimulation of Ca^{2+} oscillations and airway contraction, in the absence of contractile agonist, by the release of IP_3 from caged-IP_3 (5, 6, 27, 28, 41). In addition, contractile agonist-induced Ca^{2+} oscillations were inhibited by 2-APB, another IP_3R antagonist (42).

5.1.2. Contribution of ryanodine receptors

Ca^{2+} oscillations in ASMCs have also been proposed to involve RyRs based on evidence that ACh-induced Ca^{2+} oscillations in porcine tracheal SMCs were slowed or inhibited by the RyR antagonists, ryanodine, ruthenium red and caffeine (43-45). In these cells, IP_3 was found to induce a sustained increase in Ca^{2+}, a response that is not uncommon with high concentrations of IP_3. Heparin, an IP_3R antagonist only slowed Ca^{2+} oscillations. From these data it was suggested increases in $[IP_3]$ contributed to the initiation of Ca^{2+} oscillations but that ongoing Ca^{2+} oscillations were mediated by CICR via the RyR (45). A similar idea has been proposed using muscle bundles from porcine or human airways (33, 34). However, our laboratory has extensively examined the relative roles of the IP_3R and RyR in small airways of lung slices (42) and we found that ryanodine had no or little effect on MCh-induced Ca^{2+} oscillations or Ca^{2+} waves in mouse, rat and human airways (5, 42). By contrast, the Ca^{2+} oscillations were inhibited by the IP_3R antagonist 2-APB (42, 46). Non-specific actions are a major problem associated with many pharmacological tools, and while ryanodine alters RyR activity, it can also empty the internal Ca^{2+} stores and, thereby inhibit IP_3-based Ca^{2+} oscillations. The effects of other potential inhibitors of the RyR, such as tetracaine, have been investigated but these compounds were found to have non-specific effects (33, 34). The facts that ryanodine had no effect in mouse ASMCs and that the inhibitory action of ryanodine requires an open RyR, underscores the premise that the RyR was not open at any time during MCh-induced Ca^{2+} oscillations. These data indicate that agonist-induced Ca^{2+} oscillations in healthy mouse, rat and human ASMCs rely on IP_3Rs and have little requirement for RyR (27, 42).

5.1.3. Regulation of the calcium oscillation frequency

The frequency of IP_3-based Ca^{2+} oscillations is determined by IP_3 concentration and is usually assumed to remain relatively constant in response to fixed agonist concentration. However, the activity of PLC can be Ca^{2+} sensitive and a change in $[Ca^{2+}]_i$ may generate increases in $[IP_3]$ which could feed-forward to induce further changes in Ca^{2+}. Fortunately, it is possible to distinguish between Ca^{2+} oscillations utilizing stable or oscillating $[IP_3]$ by examining the response to an increase of $[IP_3]$. In ASMCs, the photolytic release of IP_3 increased the frequency of the Ca^{2+} oscillations (41). These results imply that rapid contractile agonist-induced Ca^{2+} oscillations (20 min^{-1}; induced by 1 microM MCh) of mouse ASMCs occur by inhibitory Ca^{2+} feed-back on the IP_3R rather than by altering $[IP_3]$.

Because the frequency of the Ca^{2+} oscillations is key to ASMC contraction, other parameters determining the Ca^{2+} oscillation frequency are important. When the SR Ca^{2+} store is sufficiently depleted to prevent a subsequent Ca^{2+} release, the time taken to refill the SR Ca^{2+} store can limit the Ca^{2+} oscillation frequency (47). This period is a function of the rate of the SERCA pumps and the amount of Ca^{2+} available. The plasma membrane Ca^{2+} ATPases and Na^{+}/Ca^{2+} exchangers make it inevitable that some Ca^{2+} is lost to the extracellular space during Ca^{2+} oscillations. Consequently, a Ca^{2+} influx is required to compensate in order to maintain Ca^{2+} oscillations. In addition to serving as a Ca^{2+} reservoir, the SR $[Ca^{2+}]$ also appears to influence the gating of the IP_3R. If the SR $[Ca^{2+}]$ is below a certain threshold, it is believed the IP_3R will not open.

5.2. Calcium sensitivity of airway smooth muscle cells

A key component contributing to the variation of the frequency-contractile relationship for different contractile agonists and airways of different species is the Ca^{2+} sensitivity of the ASMCs. For the most part, Ca^{2+} sensitivity of ASMCs has been considered to reflect MLCP activity (48) and two 2 major pathways leading to decreased MLCP activity have been identified; the phosphorylation and inhibition of the regulator unit (MYPT1) of MLCP by Rho-kinase or the phosphorylation of the inhibitory protein CPI-17 by protein kinase C (Figure 1). Rho-kinase and PKC are activated by Rho and DAG, respectively, as a result of contractile agonist activation of G-protein coupled receptors (49, 50). Thus, agonists induce ASMC contraction by simultaneously stimulating both an increase in MLCK activity via Ca^{2+} increases and a decrease in MLCP activity via secondary kinases (Figure 1).

Unlike changes in $[Ca^{2+}]_i$, which are readily monitored in cells, there are no probes available with which

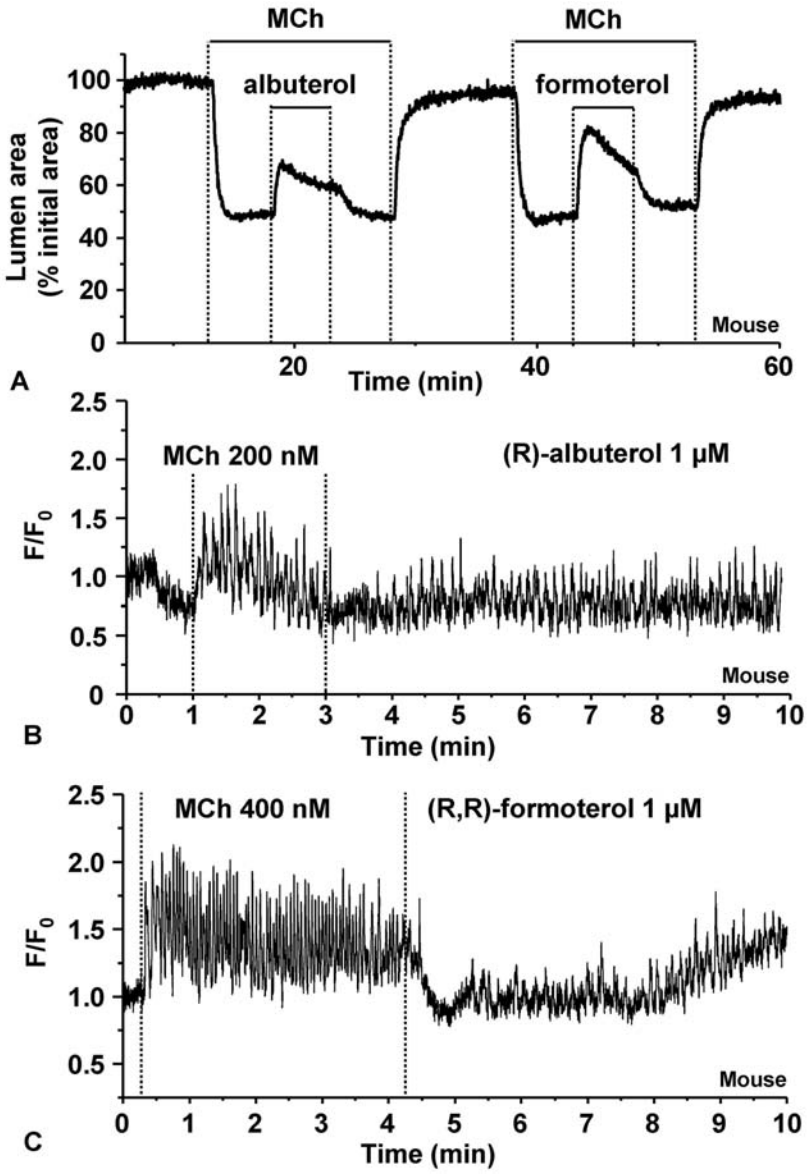

Figure 3. Effect of (R)-albuterol and (R,R)-formoterol on MCh-induced airway contraction and Ca^{2+} oscillations of ASMCs in mouse lung slices. (A) Comparison of the airway relaxation induced by (R)-albuterol and (R,R)-formoterol on the same MCh-induced contracted mouse airway. (B and C) The Ca^{2+} changes in mouse ASMCs in a lung slice following stimulation with MCh (200 or 400 nM) followed by either (B) MCh with 1 microM (R)-albuterol or (C) MCh with 1microM (R,R)-formoterol. MCh initially induced rapid Ca^{2+} oscillations (frequency proportional to concentration) which correlated with airway contraction. The addition of (R)-albuterol reduced the frequency of the Ca^{2+} oscillations and induced relaxation. The addition of (R,R)-formoterol stopped the faster Ca^{2+} oscillations induced by 400 nM MCh and induced greater airway relaxation.

to follow MLCP activity in living cells. As a result, changes in Ca^{2+} sensitivity in normal ASMCs are not easily characterized (51, 52). However, the overall Ca^{2+} sensitivity of ASMCs can be evaluated if the $[Ca^{2+}]_i$ can be experimentally held constant. To control the $[Ca^{2+}]_i$, we developed a method of Ca^{2+} permeabilization that exploits the cell's normal Ca^{2+} influx pathways (22). ASMCs within lung slices are exposed to caffeine and ryanodine to irreversibly lock the RyR in an open state. This treatment empties internal SR Ca^{2+} stores and stimulates Ca^{2+} influx, most likely via store-operated channels (SOCs).—The utilization of the RyR to make Ca^{2+}-permeabilized ASMCs seems to contradict the idea that the RyR has no role in contractile agonist-induced Ca^{2+}- oscillations. However, we have found that the RyR contributes to very slow (~1/min), non-agonist (KCl) induced Ca^{2+} oscillations (24, 28, 42). The implication is that IP_3Rs and RyRs have different roles in ASMC physiology.

A surprise revealed by Ca^{2+} permeabilized mouse lung slices was that mouse ASMCs become relaxed (transiently contract) in response to sustained increases in $[Ca^{2+}]_i$ (22).

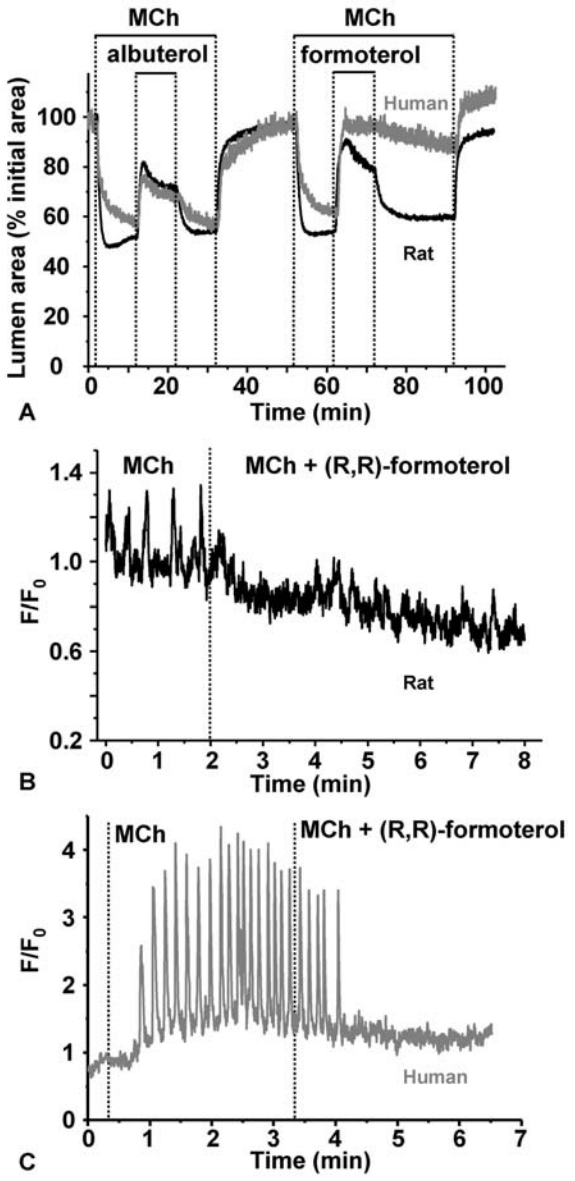

Figure 4. Effect of (R)-albuterol and (R,R)-formoterol on MCh-induced contracted airways and Ca^{2+} oscillations in rat and human lung slices. (A) Comparison of the relaxation induced by (R)-albuterol (1 microM) and (R,R)-formoterol (1 microM) on the same MCh-induced contracted airway from rat (black line) and human (grey line). The relaxation response to albuterol was fast for both rat and human airways. The reversibility of the response was faster in the rat airway. The relaxation induced by formoterol was equally fast but the reversibility of the response was extremely slow in the human airway as compared to rat. (B and C) MCh-induced Ca^{2+} oscillations were also inhibited in rat (B) and human (C) SMCs in lung slices by (R,R)-formoterol (1 microM).

However, their subsequent exposure to a contractile agonist (MCh or 5-HT) induced substantial airway contraction. Our interpretation of these results is that mouse ASMCs have an inherently low Ca^{2+} sensitivity and that contractile agonists substantially increase their Ca^{2+} sensitivity. The transient nature of the contractile response to Ca^{2+} indicates that sustained high $[Ca^{2+}]_i$ actually decreases mouse ASMC Ca^{2+} sensitivity (35). By contrast, both human and rat airways display a sustained contraction to elevated $[Ca^{2+}]_i$ and the exposure to contractile agonists further enhances this Ca^{2+} sensitivity, as judged by additional contraction (Figure 5). Consequently, in normal ASMCs, agonist-induced contraction results from a concentration-dependent change in both Ca^{2+} sensitivity and Ca^{2+} oscillation frequency. Similar conclusions were found with partial contractile agonists of the muscarinic receptor (53). Again, this comparison of the Ca^{2+} sensitivity between different species reveals substantially different responses and this probably explains the observed differences in the Ca^{2+} oscillation frequency – contractile relationships between airways of different species. In addition, this variation emphasizes that the contractile process must be examined from both a Ca^{2+} signaling and sensitivity viewpoint before it can be understood and highlights the need for care when extrapolating results from one species to another.

6. MECHANISMS OF AIRWAY SMOOTH MUSCLE CELL RELAXATION

The objective of foregoing discussion has been to understand ASMC contraction in order to identify which mechanisms are modified to mediate relaxation and to utilize these responses to counter asthmatic AHR. In the simplest terms, ASMC relaxation must result from either a decrease in $[Ca^{2+}]_i$ or Ca^{2+} sensitivity or both. The most common approach to induce airway relaxation in asthma is by increasing cAMP via the activation of adenylyl cyclase and beta2-adrenergic receptors (Figure 1).

6.1. The influence of calcium influx and calcium stores

In earlier studies, increases in $[Ca^{2+}]_i$ in ASMCs were believed to result from Ca^{2+} influx and this predicted that beta2-adrenergic compounds operated at the level of the membrane channels. A decrease in $[Ca^{2+}]_i$ could result from an inhibition of store-operated channels (SOC) (54) and an increase in Ca^{2+} efflux (55). Consistent with this idea was the finding that increases in cAMP activated BK_{ca} channels to induce membrane hyperpolarization. This would result in a decrease in Ca^{2+} influx because of the closing of voltage-dependent Ca^{2+} channels (56-58). Accordingly, iberiotoxin, an antagonist of BK_{ca} channels countered the effects of cAMP, depolarized the membrane and increased $[Ca^{2+}]_i$ (59, 60). A challenge to this hypothesis comes from the realization that agonist-induced contraction of ASMCs primarily utilizes Ca^{2+} from internal stores in the form of Ca^{2+} oscillations. Isoproterenol, forskolin and cAMP analogs were all found to induce airway relaxation while simultaneously slowing the frequency of Ca^{2+} oscillation of ASMCs in lung slices (5, 32). Similarly, the Ca^{2+} oscillation frequency was found to be decreased by beta2-adrenergic agonists in isolated tracheal cells but the effect on contraction was not reported (61, 62).

As indicated earlier, Ca^{2+} oscillations depend on the refilling of Ca^{2+} stores and this refilling can be

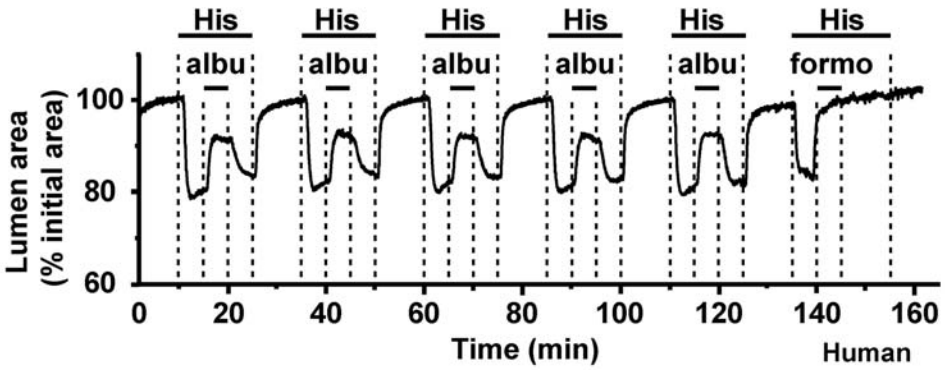

Figure 5. The effect of repetitive (R)-albuterol exposure on human airway relaxation. Representative data showing the repetitive contraction and relaxation of a human airway, as measured by a change in lumen area in response to 0.5 microM histamine (His) followed by (R)-albuterol (5 times, 1 microM, albu) and finally by (R,R)-formoterol (0.5 microM, formo). The exposure of the airway to (R)-albuterol did not change the subsequent airway relaxation induced by additional exposure to albuterol (4 times) or to formoterol.

influenced by Ca^{2+} influx, for example via SOCs (63), and this may also be a function of membrane potential. To test the idea that beta2-adrenergic agonists were reducing the frequency of the Ca^{2+} oscillations by restricting Ca^{2+} influx, the amount of Ca^{2+} available for refilling the stores was increased in several ways; these were the flash photolysis of caged Ca^{2+}, the addition of a Ca^{2+} ionophore, ionomycin, or the inhibition of BK_{ca} channels with iberiotoxin (5). Surprisingly, these treatments further slowed or even stopped the Ca^{2+} oscillations and, thereby, enhanced the relaxing effects of cAMP. These results imply that Ca^{2+} availability for store refilling was not a limiting factor. The Ca^{2+} content of the SR was also not significantly different, as judged by caffeine-induced Ca^{2+} release, in the presence or absence of forskolin, indicating that increases in cAMP were not leading to a depletion or over-filling (due to an increase in SERCA pump activity (64)) of the Ca^{2+} stores.

6.2. Sensitivity of the inositol trisphosphate receptor

The Ca^{2+} oscillation frequency is also dependent on the sensitivity of the IP_3R to IP_3. This is readily demonstrated by the photolytic release of IP_3 which induces the initiation of Ca^{2+} oscillations with a frequency proportional to the amount of IP_3 released. However, forskolin inhibited the ability of IP_3 to release Ca^{2+} in ASMCs, a result indicating that increases in cAMP inhibit IP_3-induced Ca^{2+} release from the IP_3R (5). In addition, it appears that cAMP also increases the sensitivity of the IP_3R to inhibition by Ca^{2+}; as mentioned above the Ca^{2+} oscillations were inhibited by the addition of extra Ca^{2+} in the presence of forskolin. A similar conclusion, that relaxing agonists influence internal Ca^{2+} release more than Ca^{2+} influx, was reached from studies with BK_{ca} null mice (65).

6.3. Airway relaxation induced by nitric oxide

Nitric oxide (NO) also strongly attenuates Ca^{2+} oscillations and relaxes mouse ASMCs (66, 67). In general, NO activates guanylyl cyclase to elevate cGMP and stimulate cGMP-dependent protein kinase (PKG). In other SMCs, NO-induced relaxation appears to be mediated by a decrease in $[Ca^{2+}]_i$ because of an inhibition of the IP_3R by the binding of an inhibitory protein after it is phosphorylated by PKG (named IP_3R associated protein, IRAG) (68, 69). This cGMP-associated inhibition of the IP_3R is similar to the inhibition of the IP_3-sensitivity of the IP_3R associated with increases in cAMP. However, in IRAG or PKG deficient mice, SMC relaxation still occurred in response to a cAMP analog, implying that cAMP does not affect the IP_3R via a mechanism involving the phosphorylation of IRAG. A slowing of Ca^{2+} oscillations was also induced by NO in isolated porcine tracheal cells (70) but because the Ca^{2+} oscillations in these cells was believed to rely more on RyRs, it is not clear where NO (cGMP) acts.

6.4. Airway relaxation induced by beta2-adrenergic agonists

6.4.1. Effects of beta2-adrenergic agonists on calcium oscillations

This theme of inducing airway relaxation by slowing Ca^{2+} oscillations is also displayed by the beta2-adrenergic agonists, (R)-albuterol and (R,R)-formoterol in mouse (6, 32) and human (27) ASMCs (Figure 3). At equal concentrations, formoterol was substantially more effective at inducing airway relaxation than albuterol and this was reflected in the different abilities of these relaxing agonists to slow the Ca^{2+} oscillations (Figure 3). (R,R)-formoterol (1 microM) completely abolished Ca^{2+} oscillations and induced approximately 85 % relaxation in mouse airway, whereas (R)-albuterol (1 microM) only induced a transient arrest of the Ca^{2+} oscillations. The Ca^{2+} oscillations partially recovered in the presence of albuterol. This correlates with the partial recontraction of the airway during the exposure to albuterol. However, the Ca^{2+}oscillation frequency remained reduced and correlated with a sustained airway relaxation of about 25%. (R)-albuterol- and (R,R)-formoterol also induce airway relaxation in lung slices from rat and human and this is associated with a reduction in the frequency of the Ca^{2+} oscillations of ASMCs (Figure 4).

In keeping with the idea that the IP_3R has a decreased sensitivity to IP_3 in the presence of elevated cAMP, presumably induced by albuterol and formoterol, the photolytic release of IP_3 briefly restored the Ca^{2+} oscillations in the continued presence of formoterol. However, the fact that formoterol usually abolished Ca^{2+} oscillations whereas albuterol only slowed the frequency of Ca^{2+} oscillations, suggests that, in addition to a reduction in the IP_3R sensitivity to IP_3, it seems likely that formoterol also decreased the amount of IP_3 available to stimulate Ca^{2+} oscillations. The implication is that formoterol may also exert some inhibitory action at the cell membrane to reduce PLC activity (71, 72).

6.4.2. Effects of beta2-adrenergic agonists on receptor desensitization

As mentioned earlier, a potential problem associated the use of beta2-agonists has been receptor desensitization, especially in response to isoproterenol (5, 8). However, albuterol-induced desensitization to albuterol in humans appears to be relatively weak. The relaxation induced by multiple applications of albuterol to human airways contracted with histamine remained similar (Figure 5). Furthermore, this exposure to albuterol had little effect on the subsequent action of formoterol (Figure 5). These responses are in contrast to the action of isoproterenol on mouse airways, where the response to isoproterenol was quickly attenuated (5). Cooper and Panettieri (8) also reported that albuterol induced desensitization to isoproterenol in airways humans. Interesting, the receptor desensitization to beta2-adrenergic agonists could be prevented by treatment with the glucocorticoid steroid, dexamethasone. Glucocorticoid steroids might increase beta2-adrenergic agonists receptors gene transcription and/or the coupling of the receptor to adenylate cyclase. These data support the clinical approach of a combined use of steroids and beta2-adrenergic agonists, which could offer therapeutic benefit over either therapy alone.

6.4.3. Effect of enantiomers of beta2-adrenergic agonists on airway relaxation

We also investigated the idea that S-enantiomers of albuterol and formoterol have antagonistic effects on airway relaxation induced by the R-enantiomers. Surprisingly, while racemic albuterol consistently induced less airway relaxation than (R)-albuterol, (S)-albuterol alone had no effect on airway size (32). By contrast, the relaxation induced by racemic and (R,R)-formoterol were undistinguishable in mouse small airways, but (S,S)-formoterol alone was capable of inducing a small relaxation. Yet, neither (S,S) formoterol nor (S)-albuterol had any effect on methacholine-induced Ca^{2+} oscillations (6, 32). These data imply that S-albuterol antagonizes the action of (R)-albuterol while (S,S)-formoterol may facilitate the action of (R,R) formoterol. These findings are in contrast to those obtained by Mitra *et al.*, (16) who reported that (S)- and racemic albuterol increased $[Ca^{2+}]_i$ in bovine tracheal SMCs and that, in some cells, (S)-albuterol induced multiple Ca^{2+} transients. This study also suggested that (S)-albuterol bound to muscarinic receptors to activate IP_3 production and the release of internal Ca^{2+}. The most likely explanation for these discrepancies is the difference in the techniques underlying the studies. Mitra *et al.*, (16) examined the effects of albuterol in non-contracted ASMCs rather than ASMCs exhibiting significant contraction and Ca^{2+} signaling. In addition, the ASMCs used were enzymatically isolated rather than remaining *in situ* as a part of a lung slice. Increases in $[Ca^{2+}]_i$ induced by (S)-albuterol and salbutamol have also been observed in human ASMCs, but only a single time point from a population of cells is reported (18).

6.4.4. Effects of beta2-adrenergic agonists on calcium sensitivity

The complementary mechanism to a decrease in the Ca^{2+} oscillation frequency by which beta2-adrenergic agonists may induce airway relaxation is a decrease in Ca^{2+} sensitivity. While a cAMP-dependent increase of MLCP activity is an attractive hypothesis for this decreased Ca^{2+} sensitivity, there might be other non-phosphorylation dependent mechanisms of Ca^{2+} sensitivity that may also be altered. With the use of Ca^{2+}-permeabilized lung slices, we found that treatments that increase cAMP (forskolin and phosphodiesterase inhibitors) relaxed airways by decreasing the Ca^{2+} sensitivity of mouse ASMCs (22). A similar finding was obtained from studies with guinea pig trachea (73). Consistent with these findings is the observation that (R,R)-formoterol (Figure 6) as well as (R)-albuterol induced a decrease in the Ca^{2+} sensitivity of mouse and human ASMCs. Formoterol appears to be much more effective than albuterol in this respect. At concentrations that induced equal relaxation (5 nM formoterol and 1 microM albuterol), formoterol induced relaxation primarily by decreasing Ca^{2+} sensitivity without altering the Ca^{2+} oscillation frequency (6). In addition, (S,S)-formoterol also induced a small decrease in Ca^{2+} sensitivity and, as a result, the decrease in Ca^{2+} sensitivity induced by racemic formoterol was greater than that of (R,R)-formoterol (Figure 6). By contrast, (S)-albuterol had no effect on Ca^{2+} sensitivity.

From these data, it appears that (R)-albuterol and (R,R)-formoterol induce airway relaxation by reducing both the Ca^{2+} oscillations and Ca^{2+} sensitivity of ASMCs. In both respects, formoterol has more efficacy than albuterol and induces greater airway relaxation. While (S,S)-formoterol can facilitate airway relaxation via a decrease in Ca^{2+} sensitivity, this action has little advantage for racemic formoterol when the effects of reduced Ca^{2+} oscillations and decreased Ca^{2+} sensitivity are combined. By contrast, (S)-albuterol antagonizes the relaxation induced by (R)-albuterol, but the mechanism of action does not appear to involve changes in Ca^{2+} oscillation frequency or Ca^{2+} sensitivity. Interestingly, NO or 8Br-cGMP only have a small effect on Ca^{2+} sensitivity; this implies that cGMP-dependent airway relaxation is primarily mediated by decreases in Ca^{2+} oscillations.

7. SUMMARY AND PERSPECTIVE

In general, the actions of (R)-albuterol and (R,R)-formoterol appear to be similar in human and rat airways; both beta2-adrenergic agonists induce a reduction in Ca^{2+} oscillations and Ca^{2+} sensitivity in proportion to the amount

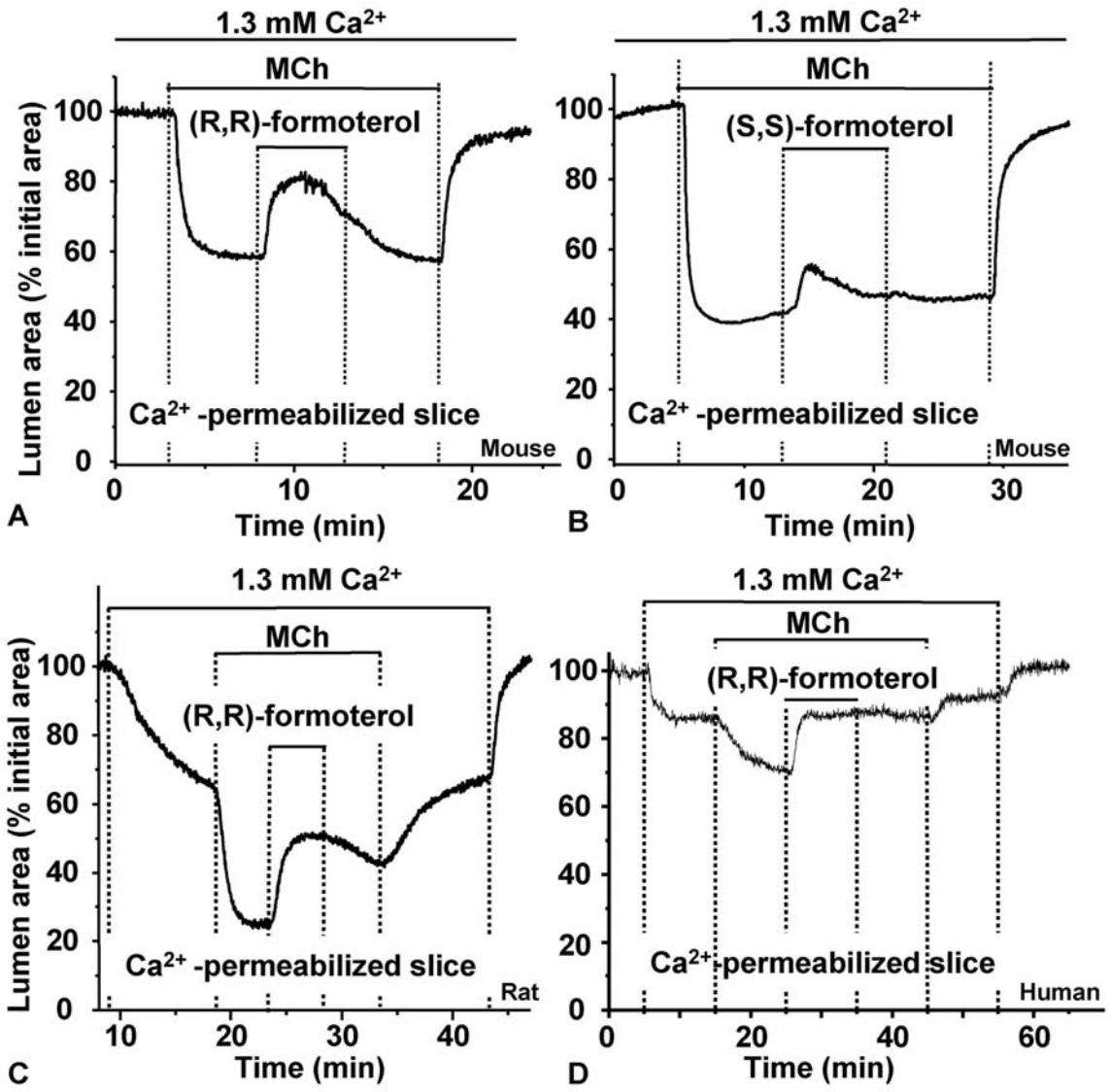

Figure 6. Correlation between airway relaxation and Ca^{2+} sensitivity induced by formoterol in mice, rat and human Ca^{2+} permeabilized lung slices. (A) Representative data showing airway contraction induced by 400 nM MCh followed by relaxation induced by (R,R)-formoterol (1 microM) in a Ca^{2+}-permeabilized mouse lung slice. Under these conditions of high extracellular Ca^{2+}, the ASMCs of Ca^{2+}-permeabilized lung slices have a high $[Ca^{2+}]_i$ but the airways of the mouse are relaxed due to a Ca^{2+}-dependent decrease in Ca^{2+} sensitivity. The addition of agonist (MCh) induced contraction without altering the $[Ca^{2+}]_{i,}$ (not shown) and this indicates an increase in Ca^{2+} sensitivity. By contrast, the addition of beta2-adrenergic agonist induced airway relaxation which indicates a decrease in Ca^{2+} sensitivity. (B) Similar data showing that (S,S)-formoterol could also decrease Ca^{2+} sensitivity but to a smaller extent. The effects of formoterol were quickly reversed in mouse airways. (C and D) Representative data showing that (R,R)-formoterol (1 microM) also decreased Ca^{2+} sensitivity in Ca^{2+}-permeabilized rat (C) and human (D) lung slices. In contrast to mouse airways, the increase in $[Ca^{2+}]_{i,}$ resulting from the addition of extracellular Ca^{2+}, induced contraction in rat and human lung airways. This response indicates that rat and human airways are inherently more Ca^{2+}-sensitive than mouse airways. A further airway contraction indicating an increase in Ca^{2+} sensitivity was induced by MCh (500 nM). The exposure to formoterol relaxed the airways indicating a decrease in Ca^{2+} sensitivity. The reversibility of this relaxation was slow in rats and substantially delayed in human airways.

of airway relaxation. However, the dynamics of these changes are significantly different (Table 1). (R,R)-formoterol is defined as a LABA, and as such, we expected it to have, like salmeterol, a slow onset and offset of action. In mouse airways, the actions of albuterol (a SABA) and formoterol are both fast and readily reversible (Figure 3). On the other hand, the onset of action of formoterol in rat and human ASMCs is slower. More notably, the offset of formoterol action is delayed in rats but excessively prolonged in human ASMCs (Figures 4 and 5) (27). This difference in

Table 1. A general relative summary of the physiological responses of airway smooth muscle cells from different species

	Species		
Physiological Response[1]	***Mouse***	***Rat***	***Human***
Ca^{2+} oscillation frequency	Fast	Slow	Slow
Ca^{2+} sensitivity	Low	High	Medium to High
Response to Isoproterenol			
relaxation	Small, transient, Fast		
recontraction	Fast		
Ca^{2+} oscillations	Slowed		
Ca^{2+} sensitivity	Decreased		
Desensitization	Fast (with minutes)		Strong
Response to Albuterol (R)			
relaxation	Small, Fast	Small, Fast	Small, Fast
recontraction	Fast	Fast	Medium
Ca^{2+} oscillations	Slowed	Slowed	
Ca^{2+} sensitivity	Weakly inhibited	Partially Inhibited	
Desensitization	Not observed[2]	Not observed[2]	Not observed[2]
Response to Formoterol (RS or RR)			
relaxation	Large, Fast	Large, Fast	Large, Fast
recontraction	Fast	Medium	Very Slow
Ca^{2+} oscillations	Stopped	Stopped	Stopped
Ca^{2+} sensitivity	Strongly Inhibited	Strongly Inhibited	Fully Inhibited
Desensitization	Not observed[2]	Not observed[2]	Not observed[2]

[1]To similar concentrations of the same stimulating agonist (usually MCh), [2]During our experiments which lasted for several hours (see fig 5), For quantification details, refer to 5, 6, 8, 22, 24, 27, 28, 32. Blank entry indicates we have no data available.

reversibility between mouse and human airways argues against the non-specific hypothesis of action that cell membranes simply accumulate the lipophilic formoterol for a subsequent slow release. It would seem more likely that formoterol has a higher binding affinity to the beta2-adrenergic receptors of rats and humans. In view of these long-lasting actions, it will be interesting to determine the effects of the S-enantiomers in human airways.

These results also raise the question of the validity of simply extrapolating data from one species to another. The use of mouse lung slices has provide invaluable insights and techniques to study the contractile mechanisms of ASMCs, and, it is now rapidly becoming evident that these approaches must be complemented, by comparative studies of human lung slices.

8. ACKNOWLEDGMENTS

This work was supported by grants from NIH (HL71930 and HL087401) and Sepracor Inc.

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Abbreviations: histamine (His), methacholine (MCh), potassium (KCl), leukotriene D4 (LTD_4), airway smooth muscle cells (ASMCs), myosin light chain kinase (MLCK), myosin light chain phosphatase (MLCP), inositol 1,4,5 trisphosphate (IP_3).

Key Words: Albuterol, Formoterol, Phase Contrast Microscopy, 2-Photon, Calcium Sensitivity, Calcium Oscillations, Lung Slice, Review

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