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Second messengers, ion channels and pharmacology of airway smooth muscle

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ABSTRACT: The airway smooth muscle cell is the chief effector cell governing the control of airway calibre in the human lung. The contractile state of the airway smooth muscle cell is predominantly influenced by the balance of constrictor and relaxant stimuli.

Agents such as histamine and acetylcholine cause airway smooth muscle cells to contract through activation of specific cell surface receptors and engagement of signal transduction pathways and/or ion channels. The predominant pathway mediating constriction is activation of phospholipase C, with release of inositol 1,4,5-triphosphate and elevation of intracellular calcium levels.

Relaxation is brought about predominantly by stimulation of adenylyl cyclase-coupled receptors (*e.g.* the β_2-adrenoceptor) resulting in elevation of cell cyclic adenosine monophosphate content. Complex crosstalk occurs between both of these pathways and also ion channels expressed on the airway smooth muscle cell membrane, leading to careful regulation of airway smooth muscle tone.

A greater understanding of the mechanisms governing control of these pathways will lead to the identification of novel therapeutic targets which will in turn lead to new agents for the treatment of asthma.

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The airway smooth muscle cell is critically important in asthma, mediating not only the bronchoconstrictor effects of agents such as histamine and methacholine but also the bronchodilator effects of β_2-agonists. In principle, an understanding of the mechanisms underlying control of the airway smooth muscle cell is important in defining the pathophysiological abnormalities present in asthma and other airway diseases. Both the contractile and relaxant responses of airway smooth muscle are regulated by crosstalk between the important intracellular signalling pathways controlling contraction and relaxation. This review summarizes the key processes involved in the regulation of these two responses in airway smooth muscle. Although many of these pathways are complex, it is important that understanding of these processes is increased. Most therapeutic agents currently in use as bronchodilators interact with these pathways, and novel targets exist which can potentially be exploited in the development of new asthma therapies.

Model systems

Before discussing the regulation of contractile and relaxant responses in airway smooth muscle in detail, it is important to consider the model systems in which these processes have been studied. Obviously, the most relevant system to patients with respiratory disease is tissue derived from human subjects. However, limited amounts of tissues (especially from asthmatic individuals) have been available and hence there are few published data on human airway smooth muscle *ex vivo*. The majority of published data involve either *ex vivo* studies of airway smooth muscle from animals (*e.g.* guinea-pig or bovine trachea) or, more recently, airway myocytes in primary culture. Both of these systems have potential drawbacks. Although experimental animal airway smooth muscle has many similarities to human airway smooth muscle, there are subtle differences, *e.g.* in the receptor subtype expressed or in the relative balance between the inflammatory mediators present in

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preparations. Conversely, using cultured airway smooth muscle, which allows human tissue to be utilized, imposes a different series of problems because of the dedifferentiation that occurs during cell culture [1]. Thus, following subculture, human airway smooth muscle cells show reduced expression of contractile protein elements [2], a number of important ion channels and some receptor subtypes (*e.g.* the muscarinic M_3 receptor) [3], whereas other receptors (*e.g.* prostanoid receptors) appear to be upregulated in cultured cell systems [4]. Recently, a number of groups have attempted to redifferentiate airway smooth muscle by altering culture conditions, often utilizing extended periods of growth arrest [5, 6]. Preliminary data suggest that this may result in a return to a more differentiated phenotype. However, to date, the majority of published data using cultured airway smooth muscle have been performed on cells which are at least partially dedifferentiated and which perhaps would be best called airway myofibroblasts.

Contractile responses

Following stimulation of airway smooth muscle by classical contractile agonists such as histamine or methacholine, initiation of the contractile response depends upon stimulation of phospholipase C-dependent pathways [7, 8] (fig. 1). These pathways have been extensively studied in cultured and noncultured airway myocytes from a range of species, although, because of difficulty in obtaining adequate amount of tissue, the majority of human studies have been performed using cultured cell systems [9]. The key intracellular signalling steps involved in the contractile response are shown in figure 1 using histamine as a paradigm. Histamine binds to the histamine H_1 receptor present on the cell surface: this receptor is a member of the seven transmembrane domain G protein-coupled receptor superfamily which is responsible for the action of most important agonists in airway smooth muscle [10]. Binding of histamine to the H_1 receptor results in stimulation of the associated G protein, G_q, which dissociates releasing the active α subunit which is able to stimulate phospholipase C. This enzyme stimulates the breakdown of the membrane phospholipid phosphatidyl inositol 4,5 bisphosphate (PIP_2), resulting in the production of the two intracellular second messengers inositol 1,4,5 triphosphate (IP_3 and diacylglycerol (DAG). IP_3 diffuses through the cytosol and binds to specific sites (the IP_3 receptor) on intracellular calcium stores, resulting in release of calcium from the intracellular stores into the cytoplasm. This produces a brief rise in the intracellular free calcium concentration. DAG, the other product of PIP_2 hydrolysis, is able to activate protein kinase C, which in turn can phosphorylate key targets, resulting in an altered sensitivity of the contractile apparatus to calcium. Recently, it has also been suggested that DAG may have an additional role as an agonist for nonvoltage-dependent calcium channels present in the tissue.

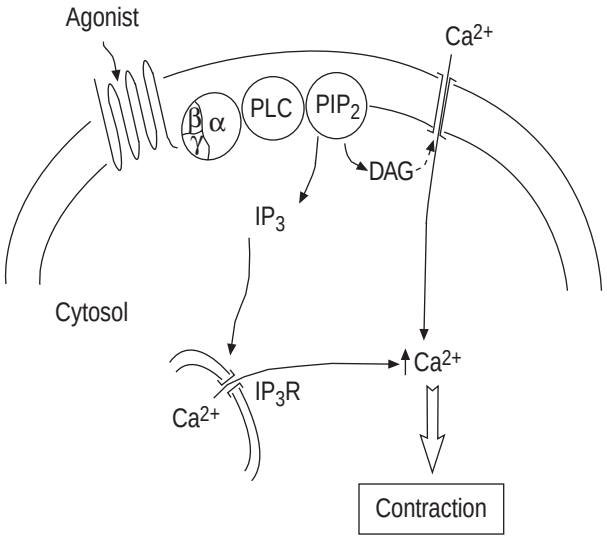

Fig. 1. – Pathways involved in airway smooth muscle contraction. Following binding of agonist to receptor in the cell membrane, the associated G protein which exists as a heterotrimeric complex of α, β and γ subunit dissociates: the free α subunit stimulates phospholipase C (PLC) which in turn catalyses the breakdown of phosphatidylinositol 4,5 biphosphate (PIP_2). This results in the formation of the two intracellular messengers diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3). IP_3 is able to release calcium from intracellular stores *via* the IP_3 receptor (IP_3R), whereas DAG, in addition to activating protein kinase C (not shown), may also be able to stimulate calcium entry. The rise in cytosolic free calcium levels leads to contraction of the airway smooth muscle cell.

A number of other contractile agonists coupled to receptors expressed on airway smooth muscle are also able to stimulate contractile responses through the same intracellular signalling pathway. These agonists, and, where characterized, the receptors through which they operate, are shown in table 1.

Control of intracellular calcium concentration

If the intracellular calcium response to an agonist such as histamine is followed in freshly isolated airway smooth muscle, cultured airway smooth muscle or permeabilized strips of tracheal muscle, a characteristic profile of intracellular calcium levels following agonist stimulation is observed (fig. 2) [20]. Experiments designed to study intracellular calcium signalling have utilized a range of calcium-sensitive fluorescent probes which can be trapped inside the cell such as Fura 2 and Fluo 3. Combining the use of cells "loaded" with these agents with sophisticated imaging methods allows changes in calcium levels in single living cells to be followed in "real time" [18, 21]. Following stimulation of a cell with agonist, an initial rapid rise in intracellular calcium levels occurs. This reaches a maximum within 10–15 s and then rapidly declines

Table 1. – Contractile agonists in airway smooth muscle and their receptors

[Ref.]	Agonist	Receptor	Comments
[10]	Histamine	H_1	
[11–14]	Acetylcholine	M_3	M_2 also present, role unclear
[15]	Leukotriene D_4	LTD_4	
[16]	Substance P	?NK_2	
[17, 18]	Bradykinin	B_2	Some evidence for atypical bradykinin receptors ?B_3
[19]	5-Hydroxytryptamine	?5-HT_2	Subtype not fully defined

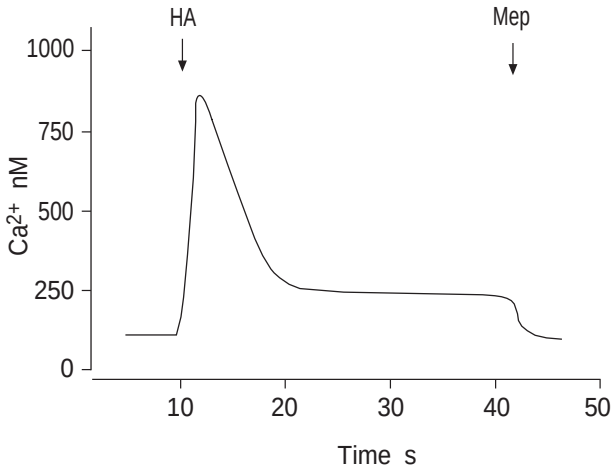

Fig. 2. – Calcium transient in Fura 2-loaded airway smooth muscle cell. Following stimulation with histamine (HA), basal calcium levels rise from ~100 nM to a peak of 0.5–1 μM before falling to a sustained plateau phase. This plateau phase is dependent upon entry of calcium across the cell membrane (see text) and the continued presence of agonist: addition of the histamine H_1 receptor antagonist mepyramine (Mep) results in a fall back to basal calcium levels.

again and is dependent upon release of calcium from intracellular stores. This is mediated by agonist-induced IP_3 production and subsequent stimulation of IP_3 receptors on the sarcoplasmic reticulum as discussed above [7]. The precise mechanism whereby calcium concentrations subsequently fall to resting levels is less clear, although reuptake into stores is the most likely explanation: calcium pumps can extrude calcium from the cell but the time course of the observed response seems too rapid for this mechanism to be important. However, in the continued presence of agonist, calcium levels do not fall completely back down to baseline levels, and a sustained plateau response is usually observed (fig. 2) [20]. This sustained calcium response is important for maintaining the contractile response to agonist. It is clear that the source of calcium for the maintained "plateau phase" of the calcium response is an influx of calcium from extracellular sources: if extracellular calcium concentrations are reduced to submicromolar levels, the plateau phase is lost. The sustained calcium entry occurs through a channel (or channels) mechanism which remains poorly characterized [20]. Two models for calcium influx have been proposed: 1) a capacitative calcium entry which is stimulated by store emptying; and 2) a noncapacitative entry pathway independent of store emptying but dependent upon receptor activation of specific channels [21]. The molecular identity of calcium entry pathways in airway smooth muscle has remained obscure, although recent data from recombinant cell systems has suggested that homologues of the *Drosophila* transient receptor potential (*TRP*) gene family may be potential candidates [22]. At present there are seven known human members of the *TRP* gene family (*HTRP*), of which *HTRP1*, *-3*, *-4* and *-6* are potential calcium entry pathways which are expressed in cultured human airway smooth muscle [23]. The recent observation that *HTRP3* can be stimulated by DAG provides an attractive mechanism for sustained calcium entry in nonexcitable cells, although whether this is important in airway smooth muscle remains to be determined, and, indeed, *HTRP4* exhibits characteristics more akin to the electrophysiological features of the putative airway myocyte channel.

Relaxation through Gs-coupled receptors

Airway smooth muscle cells express at least two receptors coupled to adenylyl cyclase *via* a stimulatory G protein (Gs), namely the β_2-adrenoceptor [4, 24, 25] and a prostanoid receptor (probably EP_2) [4, 26]: there may be, in addition, a small population of vasoactive intestinal peptide receptors, at least in some species [27, 28]. Stimulation of adenylyl cyclase-coupled receptors results in Gsα dependent activation of adenylyl cyclase in a manner analogous to activation of phospholipase C pathways: thus, following binding of an agonist such as salbutamol to the β_2 adrenoceptor, Gs dissociates and the active α subunit of Gs stimulates adenylyl cyclase activity (for review, see [29]). Adenylyl cyclase catalyses the breakdown of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (AMP) and cyclic AMP in turn is able to activate protein kinase A, which phosphorylates key intracellular proteins and thus is responsible for the majority of the physiological response to stimulation of β_2-adrenoceptors (fig. 3). Cyclic AMP is broken down by intracellular phosphodiesterases (see below). Airway smooth muscle also contains a population of muscarinic M_2 receptors which are negatively coupled to adenylyl cyclase *via* an inhibitory G protein (Gi) [30–32]. Stimulation of muscarinic M_2 receptors by acetylcholine inhibits activation of adenylyl cyclase, which results in an acute lowering of intracellular cyclic AMP levels. Thus intracellular cyclic AMP concentration is controlled by the counterbalance between activation of Gs-coupled receptors and Gi-coupled receptors together with regulation at the level of breakdown of cyclic AMP by phosphodiesterase isoforms (see below).

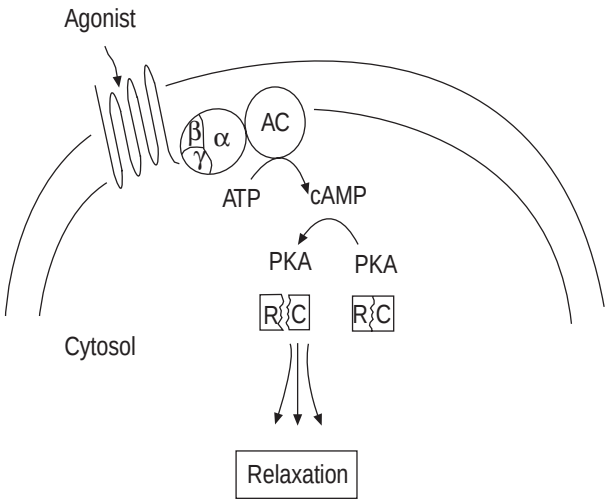

Fig. 3. – Pathways involved in airway smooth muscle relaxation. Following binding of agonist to receptor (*e.g.* the β_2 adrenoreceptor), the associated stimulatory G protein (Gs) dissociates freeing the stimulatory α subunit from β γ. Gsα is able to activate adrenylyl cyclase (AC), which catalyses the breakdown of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP); this in turn activates protein kinase A (PKA), leading to the dissociation of the catalytic subunit (C) from the regulatory subunit (R). The catalytic subunit is able to phosphorylate key targets within the cell leading to relaxation.

There are a number of targets for protein kinase A which can potentially result in relaxation of airway smooth muscle. These are summarized in table 2. Because membrane hyperpolarization causes myocyte relaxation, stimulation of the calcium-activated potassium channel (K_{Ca}, also called BK or Maxi K) results in relaxation [33]. The mechanisms underlying regulation of K_{Ca} have been dissected over the last few years and it is now clear that this channel is subject to complex regulation, being stimulated by both Gsα directly [34] and the catalytic subunit of protein kinase A [35]. In addition, K_{Ca} is inhibited by Giα directly following stimulation of musearinic receptors (presumably M_2) [36, 37]. An increase in channel activity results in membrane hyperpolarization and relaxation [38]. Whether this remains the key effector pathway for cyclic AMP/protein kinase A-mediated relaxation remains uncertain, although toxin inhibitors of this channel such as charybdotoxin and iberiotoxin potently antagonize the relaxant effect of β_2-agonists on strips of airway smooth muscle [33, 38]. Other potential targets for protein kinase A include altering the phosphorylation state of myosin light chain kinase and/or the contractile apparatus within the cell, and promoting calcium extrusion (table 2) [39, 40].

Cyclic guanosine monophosphate and airway smooth muscle

As mentioned above, agents which elevate cyclic guanosine monophosphate (GMP) levels such as sodium nitroprusside are able to bring about the relaxation of airway smooth muscle preparations in organ bath systems. The potential importance of cyclic GMP in relaxant responses in airway (as opposed to vascular) smooth muscle *in vivo* is unclear. Potential stimulants of guanylyl cyclase *in vivo* include atrial natriuretic peptide (ANP) and nitric oxide. ANP is predominantly produced in the heart, and plasma ANP levels have been shown to be elevated during exercise and acute episodes of asthma [41, 42]. Sodium nitroprusside, ANP and the related brain natriuretic peptide have all been shown to cause elevation of the cyclic GMP content of cultured human airway smooth muscle [43]. NO has also been shown to cause airway smooth muscle to relax *in vitro* [44]: NO is probably produced primarily from the epithelium following stimulation with agents such as bradykinin. In keeping with this, bradykinin is able to cause elevation of cyclic GMP levels and relaxation of guinea pig tracheal muscle in preparations containing intact epithelium but not those which are epithelium-denuded [45, 46].

The mechanisms underlying cyclic GMP-induced relaxation are less clear than those underlying cyclic AMP-induced relaxation. Cyclic GMP is able to activate cyclic GMP-dependent protein kinase (PKG), and at higher concentrations can also activate protein kinase A [47]. Cyclic GMP-dependent protein kinase is able to mimic many of the actions of protein kinase A including effects on calcium homeostasis, altering the sensitivity of contractile proteins to calcium and membrane hyperpolarization *via* activation of K_{Ca} [47–55]. There may also be indirect effects of cyclic GMP *via* its ability to inhibit the type 3 cyclic GMP-inhibited cyclic AMP-selective phosphodiesterase present in airway smooth muscle (see below). Elevation of cyclic GMP levels in airway smooth muscle occurs as a result of increased activity of guanylyl cyclase. Two different major forms of guanylyl cyclase, soluble (cytosolic) and particulate guanylyl cyclase exist. Whereas ANP interacts with the particulate form of guanylyl cyclase, NO interacts with the soluble form. Little is known about the isoforms of guanylyl cyclase present in airway smooth muscle, although the α_1/β_1 form of soluble guanylyl cyclase has been identified in bovine lung [56].

Table 2. – Mechanisms underlying airway smooth muscle relaxation by β_2-agonists

Cyclic AMP-dependent
Inhibition of inositol phospholipid hydrolysis
Stimulation of K_{Ca}
Membrane hyperpolarization
Inhibition of myosin light chain kinase activation
Alteration of phosphorylation state of contractile apparatus
Increased Ca^{2+} reuptake/extrusion
Cyclic AMP-independent
Stimulation of K_{Ca} by Gsα

AMP: adenosine monophosphate; K_{Ca}: calcium activated potassium channel; Gsα: α subunit of stimulatory G protein.

Crosstalk between signalling pathways

In addition to the responses described above, complex crosstalk occurs between signalling pathways in many cell types including airway smooth muscle [57]. For example, elevation of cellular cyclic AMP content inhibits signalling through phospholipase C pathways, thus counteracting the effect of contractile agonists such as histamine [58–61]. This inhibitory mechanism is dependent upon activation of protein kinase A and occurs at the post-receptor level, presumably at the level of phospholipase C itself [59]. However, even crosstalk is agonist-dependent: in contrast to histamine-induced PIP_2 hydrolysis, cholinergic agonist-induced inositol phosphate responses are not inhibited to any major extent by β_2-agonist-induced elevation of cellular cyclic AMP content, although the phosphodiesterase type IV inhibitor rolipram is able to inhibit this response [62]. As discussed above, stimulation of M_2 receptors inhibits cyclic AMP formation in airway smooth muscle. It can thus be seen that, in airway smooth muscle, a series of complex controlling mechanisms have evolved which have resulted in cholinergic stimulation resulting in both contraction through stimulation of phospholipase C-dependent pathways and also inhibition of relaxation through acute inhibition of adenylyl cyclase (and thus inhibition of cyclic AMP-dependent relaxation pathways). Even this may be more complex than initially thought: recent data have suggested that prolonged stimulation of muscarinic M_2 receptors may in fact sensitize adenylyl cyclase and thus the M_2 receptor present on airway smooth muscle may subserve two roles, with acute inhibition of adenylyl cyclase but long-term homeostatic regulation serving to upregulate adenylyl cyclase expression and thus counteract the action of muscarinic agonists at M_3 receptors coupled to phospholipase C [63].

Phosphodiesterases

Although theophylline has been used clinically for many years and is a relatively weak relaxant of airway smooth muscle preparations in organ bath systems, it has recently become clear that many different isoforms of phosphodiesterase exist. Molecular cloning approaches have revealed the existence of at least seven phosphodiesterase families, each family containing a number of variants, some resulting as a consequence of alternative splicing of gene family members [64]. In airway smooth muscle, the most important phosphodiesterase isoforms appear to be members of the type III and type IV families [4, 65–67]. Physiological control of cellular cyclic AMP content (by regulating cyclic AMP breakdown) appears to be predominantly due to the type IV isoform with a variable contribution from type III phosphodiesterase dependent upon the species studied. The two families can be distinguished by their regulatory characteristics: whereas both the type III and type IV phosphodiesterase families are relatively cyclic AMP-selective, members of the type III phosphodiesterase family are stimulated by cyclic GMP. As discussed above, relatively little attention has been paid to cyclic GMP as a mediator of relaxation in airway smooth muscle (as opposed to vascular smooth muscle, in which cyclic GMP has a clear-cut role).

Ion channels in airway smooth muscle

Airway smooth muscle cells exhibit marked stability of the membrane potential, which is dependent predominantly on the activity of a range of Ca^{2+}, K^+ and Cl^- channels. In some species (including human), slow wave activity is observed.

Calcium channels

There are a number of forms of voltage-dependent calcium channel which differ in their selectivity for divalent cations and their sensitivity to antagonists. Of the five main families (T, L, M, R and P/Q), the most important in most smooth muscle types is probably the L type channel. The channels are formed by hetero-oligomeric complexes consisting of an α_1 subunit which forms the pore of the channel and provides the extracellular binding site for most agonists and antagonists together with a β, α_2-δ and possibly γ subunit. Although voltage-dependent calcium channels can be readily identified in freshly isolated airway myocytes and channel activity is increased by cholinergic agents such as methacholine, the overall contribution of these channels to agonist-induced contractile responses seems small. As mentioned above, the prolonged rise in intracellular calcium levels seen following stimulation with agonists such as histamine is insensitive to classical voltage-dependent calcium channel antagonists, in addition, these agents are poor bronchodilators [68–72].

Chloride channels

A large number of chloride channels have now been identified and these fall into three major groupings, voltage-sensitive chloride channels (CIC-2 to -5), volume-regulated chloride channels and another group including the cystic fibrosis transmembrane conductance regulator and the calcium-activated chloride channel. Relatively little work has been carried out on chloride channel expression in airway smooth muscle, although airway smooth muscles express a calcium-activated chloride current. These channels activate briefly following intracellular calcium release but are rapidly phosphorylated by calcium/calmodulin-dependent protein kinase, thus leading to uncoupling of the channel from regulation by cytosolic calcium [73].

Potassium channels

As has already been mentioned, potassium channels are also likely to be important in the control of membrane potential in airway smooth muscle cells [74, 75]. Under resting conditions, the membrane potential of airway smooth muscle cells is remarkably constant due to strong outward rectification. This is probably due to the presence of a delayed rectifier channel [76]. In addition, following stimulation with agents able to cause elevation of intracellular cyclic AMP levels, the high-conductance calcium-sensitive potassium channel is activated: as mentioned above this is likely to be important in producing at least part of the relaxant response to β_2-agonists [33–35]. ATP-sensitive potassium channels (K_{ATP}) are also present in airway smooth muscle, although the lack of effectiveness of activators of K_{ATP} such as levcromakalin or antagonists of K_{ATP} such as glibenclamide *in vivo* on airway tone imply that K_{ATP} is unlikely to be critically important in the regulation of contractile or relaxant responses in airway smooth muscle [77]. Recent molecular studies on the nature of the potassium channel subunits expressed in airway myocytes will help in defining the relative importance of these different channels [78, 79].

Other channels

A large number of sodium channels have been identified but relatively little information is available regarding their role (if any) in nonexcitable cells such as airway smooth muscle. In addition, a nonselective cation current has been demonstrated in airway myocytes [80] but its physiological significance remains uncertain.

Conclusion

The control of the contractile apparatus in a single airway myocyte is a complex process involving multiple conflicting signals *in vivo*. In order to achieve regulation of these influences, airway myocytes have developed a series of complex homeostatic mechanisms involving signalling through phospholipase C, adenylyl cyclase and ion channel pathways. Crosstalk between these pathways is also important in achieving homeostasis. Understanding these processes is critically important. All of the currently available bronchodilator agents produce their effects by interaction with either receptors coupled to signalling pathways or the intracellular signalling pathways themselves. However, a number of targets (for example the nonvoltage-dependent calcium entry mechanism in airway smooth

muscle) exist for which no adequate therapeutic agents are currently in use. A fuller understanding of the importance of these pathways will potentially lead to the development of novel therapeutic agents for the treatment of diseases such as asthma.

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