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Phenotypic diversity and molecular mechanisms of airway smooth muscle proliferation in asthma

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Phenotypic diversity and molecular mechanisms of airway smooth muscle proliferation in asthma. S.J. Hirst, T.R. Walker, E.R. Chilvers. ©ERS Journal Ltd 2000.
ABSTRACT: Chronic persistent asthma is characterized by poorly reversible airflow obstruction and airways inflammation and remodelling. Histopathological studies of airways removed at *post mortem* from patients with severe asthma reveal marked inflammatory and architectural changes associated with airway wall thickening. Increased airway smooth muscle content, occurring as a result of hyperplastic and/or hypertrophic growth, is believed to be one of the principal contributors to airway wall thickening.

In recent years, significant advances have been made in elucidating the mediators and the intracellular pathways that regulate proliferation of airway smooth muscle. The contribution that smooth muscle makes to persistent airflow obstruction may not, however, be limited simply to its increased bulk within the airway wall. Interest is growing in the possibility that reversible phenotypic modulation and increased heterogeneity of airway smooth muscle function may also be a feature of the asthmatic airway.

This review focuses on possible mechanisms controlling smooth muscle phenotype heterogeneity as well as on the mediators and intracellular pathways implicated in its cellular proliferation. Particular attention is paid to mechanisms involving activation of the extracellular signal regulated kinase-, protein kinase C- and phosphoinositide 3-kinase-dependent pathways, since these appear to be the major candidate second messenger pathways for G protein- and tyrosine kinase-coupled receptor-stimulated proliferation.
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Airway smooth muscle remodelling in asthma

Patients with long-standing and severe asthma often develop poorly reversible airway obstruction that is refractory to bronchodilatory and anti-inflammatory medication [1, 2]. This complication is associated with the development of persistent structural changes in the airway wall [3, 4]. These include remodelling of tissue elements in the basement membrane and in the airway tissue in general. Of these, the most striking is an increase in mural smooth muscle content. The first detailed report describing this increase in muscle was made >75 yrs ago [5]. More than 50 yrs later, several independent groups of investigators confirmed that increased airway wall smooth muscle content was a prominent pathological feature and major component of the structural changes that result in airway luminal narrowing in chronic severe asthma [4, 6–9]. However, at that time, only a handful of reports had examined the anatomical distribution of such changes, and the extent to which hyperplasia (increased cell number) and/or hypertrophy (increased cell size) contributed to the increase in muscle mass remained uncertain. In order to resolve this, EBINA *et al.* [10], using sophisticated three-dimensional morphometry, mapped these changes in both large and small airways and found that, in asthmatic subjects, airway wall thickening was present throughout

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the entire range of the bronchial tree including the smaller airways. In a subsequent study, these authors investigated the mechanism of muscular thickening and reported at least two patterns of smooth muscle thickening in the lungs of patients with fatal asthma, described as types I and II [11]. In the type I subgroup, smooth muscle cell hyperplasia was responsible for airway smooth muscle thickening in the large central airways. No hypertrophy was recorded at any level of the bronchial tree in these patients. In the type II subgroup, however, in addition to the presence of comparatively mild hyperplasia in the larger airways, cellular hypertrophy of airway smooth muscle was detected throughout the whole bronchial tree, and was particularly severe in small peripheral airways. In the absence of clinical correlates, such data may reflect heterogeneity in the processes that lead to airway wall thickening in asthma. This could involve fundamental differences in the growth-promoting mechanisms or stimuli which predominate at the smooth muscle at different levels of the bronchial tree, or, indeed, as discussed later, be related to some intrinsic heterogeneity in the nature, innervation or responsiveness of the smooth muscle itself at different levels of the bronchial tree. Although other components of airway wall remodelling may also be important in determining airways hyperresponsiveness (*e.g.* increased mucosal thickness and increased adventitial thickening), this study [11], as well as that of HEARD and HOSSAIN [6], forms the basis of the current belief that the major smooth muscle abnormality that contributes to the development of airway wall thickening in chronic severe asthma involves excessive muscle hyperplasia and hypertrophy. Accordingly, investigators are now addressing the cellular and molecular mechanisms which drive the increase in muscle in this tissue, although, to an extent, this has been at the expense of investigating other aberrant responses of smooth muscle such as increased cell survival or deposition of extracellular matrix proteins within the myobundles [12]. Conceivably, each of these processes may operate to varying degrees in chronic severe asthma, and further studies are required to determine their relative importance to the real or apparent increase in airway wall smooth muscle content that is observed *in vivo*.

Heterogeneity and phenotypic modulation of airway smooth muscle

Heterogeneity of smooth muscle function and responsiveness is rapidly emerging as an important phenomenon, particularly in disease. A broad heterogeneity exists between proximal and distal airways in receptor repertoire, innervation and in the pharmacological, electrophysiological and mechanical properties of smooth muscle [13–16]. Most recently, at the tissue level, MA *et al.* [17, 18] demonstrated clear mechanical and morphological heterogeneity of canine airway smooth muscle that is also reflected at the cellular level. Cluster analysis revealed that this heterogeneity could be divided into two broad groups: extrapulmonary and intrapulmonary airways. The extrapulmonary group (trachea and first and second bronchus generations) comprised tissue strips possessing a greater velocity of shortening and maximum shortening capacity compared to the intrapulmonary group (third to sixth bronchus generations). Based on this classification, the authors reasoned that the smooth muscle in the two groups comprised different types of smooth muscle cell. Morphological examination and electrical field stimulation of single smooth muscle cells that were enzymatically dispersed from each group, confirmed heterogeneity of smooth muscle mechanical performance at the cellular level and again revealed two groups.

In addition to heterogeneity between distal and proximal airways, heterogeneity of myocytes at a single site may also be a feature in the airway wall. For example, in the wall of the pulmonary artery, the media contains at least four phenotypically heterogenous populations of multifunctional smooth muscle cells [19]. Similarly, adult systemic arteries comprise two morphologically, ultrastructurally, biochemically and pharmacologically distinct populations of smooth muscle cells. The majority are of an adult phenotype and are located in the media, being elongated and expressing smooth muscle contractile proteins such as smooth muscle myosin heavy chains (MHCs) and smooth muscle α-actin [20, 21]. The remainder occur in the intimal layer immediately below the endothelium and are of an immature phenotype and stain for nonmuscle-MHCs and smooth muscle α-actin, with only weak expression of smooth muscle-MHCs. In clonal cell culture, these immature cells synthesize extracellular matrix proteins and proliferate in the absence of serum due to autocrine growth factor production [14, 21–23]. A similar heterogeneity is likely in other smooth muscles and evidence for this in airway smooth muscle is already emerging. HALAYKO *et al.* [24], using flow cytometric analysis of contractile protein and deoxyribonucleic acid (DNA) content in smooth muscle cells that were freshly dissociated from canine cervical trachealis, reported two populations of cells: the majority (87%) were characterized by diploid DNA content and high levels of smooth muscle myosin or α-actin content; the remainder had a tetraploid DNA content and lower contractile protein content. These and other data [13–18] clearly demonstrate the existence of smooth muscle cell heterogeneity at a biochemical, functional and morphological level even in mature healthy airways, perhaps reflecting the presence of subpopulations of cells that are at different points within their maturation programme. Because of the difficulties in obtaining airway smooth muscle from normal or asthmatic subjects, the significance of this heterogeneity in the intact human airway has not been evaluated. It may be important in determining nonhomogeneous bronchoconstriction [10, 25] or hyperplasia and hypertrophy [11] of airway smooth muscle, and has led to speculation that, in airways of individuals with chronic severe asthma, the extent of this heterogeneity may be increased, subtly altering the properties of the tissue [26, 27]. This, in turn, could offer prospects for the development of future therapeutic strategies targeting the smaller airways of the lung.

To address these and other related questions, investigators have employed cell culture systems [27–29]. The ability of isolated or cultured smooth muscle cells to contract or relax in response to specific agonists is well documented [30, 31] and airway smooth muscle cells are no exception. AVNER *et al.* [32] reported that subconfluent primary cultures of canine tracheal smooth muscle cells contracted in response to the cholinomimetic, carbachol. Maintenance of this contractile phenotype in cultured smooth muscle cells is dependent upon a number of

carefully controlled cell culture conditions, specifically cell density, avoidance of foetal bovine serum exposure and a requirement for heparin [14, 33]. Although modulation of the contractile phenotype and its role in systemic vascular disease is the subject of intense research by vascular biologists [34, 35], little attention has been paid so far to its occurrence in airway disease, although some investigators have drawn parallels in the mechanisms for vascular and airway remodelling [26, 27, 36]. Nevertheless, there is increasing acceptance that, in primary culture, exposure of airway smooth muscle cells to a mitogenic stimulus (*e.g.* serum) results in a reversible modulation in phenotype from contractile to synthetic [30, 37] that is characterized by the loss and subsequent re-expression of specific contractile phenotype markers [38, 39], as outlined in figure 1 and table 1. Contractile cells are characterized by a high density of contractile proteins and few biosynthetic intracellular organelles, are mitotically quiescent and retain their ability to contract in response to spasmogens. Synthetic smooth muscle cells have a low density of contractile proteins and high fraction of biosynthetic organelles, are mitotically active and may lose their ability to contract [30, 37]. It is probably incorrect, however, to consider these states as mutually exclusive. It is more likely that many cells have an intermediate phenotype, retaining both contractile and synthetic properties (table 1). Thus some smooth muscle cells, although still contractile, will be capable of other functions such as cell division and the production and release of pro-inflammatory mediators and extracellular matrix elements [31]. Identification of a single discriminating marker that is indicative of a particular phenotype is therefore unlikely. Indeed, with the possible exception of smooth muscle-specific MHC isoforms, no single marker is expressed unequivocally in smooth muscle [37]. Thus, classification of cell type is dependent upon the overall pattern of protein expression and intracellular structures rather than reliance on any single marker (table 1).

Although it is generally accepted that, in the airway wall, the function of the contractile smooth muscle phenotype is dedicated to the regulation of airway calibre, the existence and function of the synthetic phenotype is less clear. In systemic vascular disease, the synthetic smooth muscle cell is believed to be central to the pathogenesis of the repair injury response of an atherosclerotic lesion [34], and is responsible for the production of paracrine and autocrine factors and the synthesis and deposition of extracellular matrix components [37]. Substantial evidence from *in vitro* cell culture-based systems (discussed in detail elsewhere [26, 27, 40, 41]) now exists to support the possibility that airway smooth muscle is likewise an important source of pro-inflammatory and broncho-protective mediators, particularly in asthma. The presence of such a functionally altered phenotype may be of particular relevance in the diseased lung, in which the mass of airway smooth muscle as a fraction of the total cells in the airway wall is already increased. However, unequivocal demonstration in airway disease of this smooth muscle phenotype that is also secretory remains to be established.

Data from cell culture-based studies have identified a third possible phenotype of airway smooth muscle (fig. 1 and table 1). This is evident following prolonged withdrawal of serum (up to 20 days) from primary cultures of confluent canine tracheal smooth muscle. A subset of these cells (~20–25% of the total) reacquire the contractile protein content, shortening velocity and elongated morphology (maximal after 15–18 days of serum deprivation) that is reminiscent of contractile cells derived from the original intact tissue [28, 29]. Flow cytometric examination of their DNA content, by staining with propidium iodide, confirmed that these cells do not proliferate (S.J. Hirst, E.S. Rector and N.L. Stevens, unpublished observations). Further investigation revealed that this contractile phenotype lacked expression of SM-B, a MHC isoform thought to be important in determining smooth muscle cycling velocity, which was present in freshly isolated contractile trachealis cells [17]. Surprisingly, these contractile cells possessed a 30-fold increased content of smooth muscle myosin light chain kinase, which may substitute for the loss in SM-B, and shortened faster and to a greater extent than their freshly dispersed counterparts. Hence these cells appear to be both biochemically and functionally hypercontractile. In a subsequent study, these cells were found to express other features characteristic of contractile smooth muscle cells in the intact tissue [29]. These included expression of cholinergic M_3 muscarinic receptors and ultrastructural features such as gap junctions, myofilament stress fibres and caveolae [29]. Repeated cycles of exposure to serum followed by removal result in initial loss of this phenotype but subsequent recruitment of larger numbers of cells to this phenotype. Thus the cells appear to display true plasticity, able to express in a reversible manner features common to both contractile and synthetic smooth muscle cells, depending on the presence of serum. However, based on these functional and biochemical observations, these cells are clearly distinct from the contractile phenotype found in intact mature healthy tissue, perhaps reflecting some divergence, in which the synthetic phenotype is an intermediate step (fig. 1), from the differentiation programme that would normally occur in healthy tissue. Whether airway smooth muscle cells cultured from other species including the human can be recruited or progress through to this hypercontractile phenotype, or whether such a phenotype is an important feature of the remodelled hyperresponsive airway *in vivo*, remains to be determined. Indeed their appearance in the remodelled airway could easily go undetected unless specific markers are identified which distinguish them from otherwise normal contractile smooth muscle cells.

Fig. 1. – Diagrammatic representation of the phenotypic heterogeneity of airway smooth muscle.

Thus, the contribution that increased airway wall smooth muscle content makes to the pathogenesis of chronic asthma may not be limited to simple geometric obstruction due to airway wall thickening, but may also involve reversible phenotypic modulation from a contractile to a more synthetic/proliferative state, in which additional functions of airway smooth muscle such as production and release of pro-inflammatory mediators and extracellular matrix elements are more apparent [27, 41]. Such phenotypic modulation may result in the appearance of poorly contractile synthetic, as well as hypercontractile, smooth

Table 1. – Phenotypic heterogeneity of airway smooth muscle. Summary of the growth, ultrastructural, mechanical and biochemical features identified *in vitro*, which distinguish the contractile, synthetic and hypercontractile phenotypes of airway smooth muscle

	"Contractile"	"Synthetic"	"Hyper-contractile"
Proliferation	Slow	Rapid	Slow
Ultrastructure			
Myofilaments	Present	Reduced/ absent	Present
Golgi/RER	Present	Increased	Present
Mechanics			
(V_0/V_{max})	++	+/-	+++
Contractile proteins			
Smooth muscle α-actin	++	+/-	+++
Smooth muscle MHC	++	+/-	+++
Cytoskeletal proteins			
nm-actin isoforms (β/γ)	+/-	+++	++
nm-MHC	+/-	+++	ND
Vimentin	+	+++	ND
Regulatory proteins			
MLCK	++	+/-	++++
h-Caldesmon	++	+/-	ND
l-Caldesmon	+/-	++	ND
Calponin	++	+/-	ND
Desmin	++	+/-	ND
PKC (βI/βII)	+/-	+++	ND
CD44	+/-	+++	ND

++: expressed; +++: highly expressed; ++++: very highly expressed; +/-: poorly expressed; RER: rough endoplasmic reticulum; V_0: velocity of shortening; V_{max}: maximum shortening capacity; MHC: myosin heavy chain; nm: new muscle; *h*: heavy form; *l*: light form.

muscle cells with the overall effect of increasing, decreasing, or causing no change in smooth muscle contractility despite an increase in airway wall smooth muscle content. The cellular and molecular mechanisms which lead to these changes are now the subject of intense research, and this basic review is intended to highlight certain aspects of the current state of knowledge, as well as suggesting possible strategies for future development.

Mediators of airway smooth muscle proliferation

Most of the available studies have employed primary cell culture-based systems to elucidate the cellular mechanisms that regulate airway smooth muscle proliferation. The inherent difficulties in obtaining airway smooth muscle from asthmatic subjects and lack of suitable animal models demonstrating specific features of the remodelled asthmatic airway have hindered identification of the critical mediators and cellular mechanisms which promote airway smooth muscle proliferation *in vivo*. One promising model which satisfies some of these criteria including increased airway hyperresponsiveness and airway smooth muscle mass involves repeated ovalbumin challenge of actively sensitized Brown Norway rats [42, 43]. In studies in which the contribution of cellular hyperplasia and hypertrophy to the increased airway smooth muscle content have been investigated using this model, the number of smooth muscle cell nuclei undergoing S-phase traversal (a surrogate for proliferation), determined *in vivo* by labelling with the thymidine analogue, bromodeoxyuridine (BrdU), was found to be increased only in large- and medium-sized airways, being absent in the smaller airways [44]. The observed increase in smooth muscle content was also limited to large and medium airways, which, together with the pattern of DNA synthesis, is perhaps reminiscent of the type I thickening reported earlier by EBINA *et al.* [11] in a subset of patients with fatal asthma. Although the precise mechanisms that induce DNA synthesis in airway smooth muscle cells *in vivo* remain unknown, a leukotriene (LT) D_4 receptor antagonist, MK-571, has been shown to partially prevent the increase in airway smooth muscle content in the Brown Norway rat following repeated allergen exposure [43]. More detailed investigation using a cysteinyl LT_1 receptor antagonist, pranlukast, and a 5-lipoxygenase inhibitor prevented both DNA synthesis and the increase in airway smooth muscle content, supporting a role *in vivo* for the cysteinyl LTs in allergen-stimulated airway smooth muscle DNA synthesis in the rat [45].

Although the critical mediators and cellular mechanisms which bring about changes in airway wall smooth muscle content *in vivo* remain undefined, cell culture-based studies over the last 10 yrs have identified several putative mitogens for airway smooth muscle in human and other species. In 1990, PANETTIERI *et al.* [46] demonstrated that histamine could induce DNA synthesis and proto-oncogene expression, as well as increased cell number, in canine airway smooth muscle. The finding that histamine, a contractile agonist and classical mediator of airways inflammation, could increase airway smooth muscle growth and division prompted investigators to examine the effects of other pro-inflammatory mediators relevant to the asthma process. Various diverse stimuli have now been demonstrated to increase the proliferation of airway smooth muscle cells in cell culture, and have been reviewed in detail elsewhere [13, 47, 48]. Broadly, the substances now implicated in mitogenesis of airway smooth muscle, in addition to the action of serum itself (discussed in [49]), include pro-inflammatory mediators (histamine [46], 5-hydroxytryptamine (5-HT) [50], phenylephrine [51], tachykinins [52], endothelin (ET)-1 [53–56] and leukotriene D_4 [57]), several inflammatory cell- and plasma-derived enzymes (α-thrombin [58], tryptase [59], β-hexosaminidase and β-glucuronidase [60]), polypeptide growth factors (platelet-derived growth factor (PDGF) [61], epidermal growth factor (EGF) [62], fibroblast growth factor-2 (FGF-2) [63] and insulin-like growth factors (IGFs) [64]) and thromboxanes [65]. Pro-inflammatory cytokines (interleukin-1β [66], interleukin-6 [67], and tumour necrosis factor-α [68]) also induce a proliferative response in airway smooth muscle, which is revealed only under conditions of cyclo-oxygenase inhibition [69], in which the production and action of inhibitory prostanoids such as prostaglandin E_2 is limited. Other trophic factors such as altered mechanical stress [70] and reactive oxygen species [71] have also been identified. Components of the extracellular matrix which are increased in asthma may also impact on the proliferation of airway smooth muscle. Fibronectin and collagen I increase the sensitivity of human airway smooth muscle cells to mitogens such as PDGF-BB or α-thrombin, whereas laminin inhibits proliferation [72]. Whether or not these mediators are

important to the airway smooth muscle thickening process *in vivo* remains to be determined.

The remainder of this review examines the molecular signalling pathways that mediate mitogen-stimulated proliferation of airway smooth muscle. Specifically, it examines the growing literature implicating the extracellular signal-regulated kinase (ERK)- and protein kinase C (PKC)-dependent pathways, as well as the recently elucidated phosphoinositide 3-kinase (PI3K) pathway, as key players in the initiation and regulation of the proliferative response.

Intracellular signalling for proliferation: activation of the extracellular signal-regulated kinase pathway

The mitogens that promote smooth muscle cell proliferation fall into two broad categories: those which activate receptors with intrinsic protein tyrosine kinase (RTK) activity (*e.g.* PDGFs, EGF, FGF-2 and IGFs) and those that mediate their effects through receptors linked to heterotrimeric guanosine triphosphate (GTP)-binding proteins (G proteins) (*e.g.* α-thrombin, histamine, 5-HT, thromboxanes, ET-1 and LTD_4). A major signalling pathway activated in cultured cells by polypeptide growth factors appears to be the ERK pathway [47]. ERKs are cytosolic proline-directed, serine/threonine kinases of the mitogen-activated protein kinase (MAPK) superfamily. Three families of MAPK have been identified in various cell types. These are the c-Jun amino-terminal kinase (JNK), also known as the stress-activated protein kinase (SAPK), the p38 mammalian homologue of the yeast high osmolarity response (HOG) 1 kinase (p38MAPK) and the ERKs. ERKs are actively expressed in all airway smooth muscle systems so far tested. For maximal activation and transmission of the mitogenic signal, they require concurrent tyrosine and threonine phosphorylation of their regulatory domains. This results in their translocation to the nucleus, where they increase the *trans*-activating activity by phosphorylation of several nuclear target proteins, including the nuclear transcriptional regulatory factors, activation protein 1 (AP-1: c-Fos and c-Jun), ATF2 and Elk1. For this reason, ERKs are believed to be important in the commitment of cells to enter the cell cycle, *i.e.* transition from the G_0 to the G_1 phase. Two ERK isoforms, $p44^{ERK1}$ and $p42^{ERK2}$, are present in vascular and airway smooth muscle [47].

The classical upstream pathway leading to ERK activation requires the sequential activation of a defined protein phosphorylation cascade involving p21 Ras, Raf-1 and MAPk/ERK-activating kinase (MEK) (fig. 2). Interaction of polypeptide growth factors with their receptors results in tyrosine phosphorylation of Shc (an SH-domain containing protein and the recruitment of the guanine nucleotide-releasing-binding protein (Grb), Grb2. Grb2 localizes the guanine nucleotide exchange factor, Sos, to p21 Ras. Activation of H-, K- and N-Ras proteins, a highly conserved family of 21-kDa membrane-associated small molecular weight guanosine triphosphatase (GTPases) (*i.e.* low molecular weight G proteins) encoded by the ubiquitous H-, K- and N-ras proto-oncogenes, results in the recruitment of Raf-1, a 74-kDa cytoplasmic serine/threonine kinase. This, in turn, results in phosphorylation and activation of the MEK family of p42–45 dual specificity MAPK tyrosine and serine/threonine kinases. MEK-1

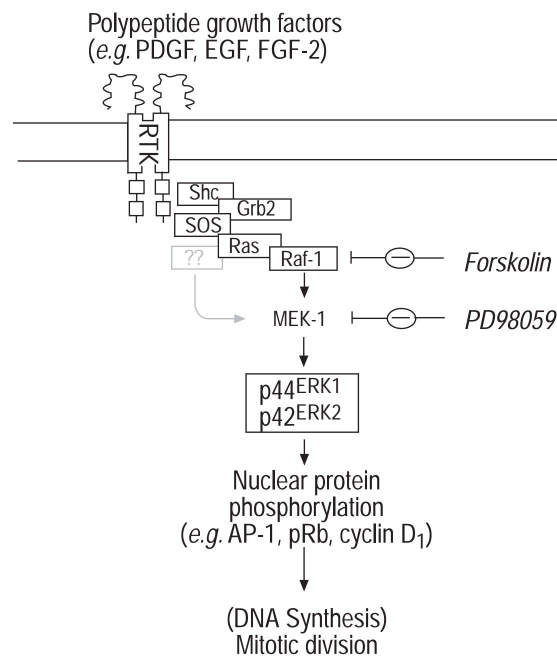

Fig. 2. – Classical extracellular signal-regulated kinase (ERK) signalling pathway for induction of deoxyribonucleic acid (DNA) synthesis in airway smooth muscle. Interaction of polypeptide growth factors with their receptors results in receptor tyrosine kinase (RTK) activation and phosphorylation of Shc an SH-domain-containing protein and the recruitment of the guanine nucleotide-releasing-binding protein (Grb), Grb2 at the membrane. Grb2 localizes the guanine nucleotide exchange factor, Sos, to p21 Ras. Activation of Ras, a 21-kDa membrane-bound low-molecular-weight G protein (with intrinsic (guanosine triphosphatase) activity), results in recruitment to the membrane of Raf-1, a 74-kDa cytoplasmic serine/threonine kinase. Activation of Raf-1 results in phosphorylation and activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)-activating kinase (MEK)-1, a member of the p42–45 kDa dual specificity MEK MAPK kinases capable of phosphorylating both tyrosine and serine/threonine residues. Activated MEK-1 directly phosphorylates (on tyrosine and threonine residues) and activates both of the ERK isoforms $p44^{ERK1}$ and $p42^{ERK2}$ which translocate to the cell nucleus where they can phosphorylate and activate various transcription factors such as the constituent regulatory elements of activation protein 1 (AP-1), retinoblastoma protein (pRb) and cyclin D_1. A Raf-1-independent pathway to ERK activation that is mediated by an unknown MEK-1 activator, proposed by HERSHENSON and coworkers [50, 73], is shown in grey Pharmacological inhibitors are shown in italics. PDGF: platelet-derived growth factor; FGF-2: fibroblast growth factor-2; EGF: epidermal growth factor.

activates both $p44^{ERK1}$ and $p42^{ERK2}$ by direct phosphorylation of tyrosine and threonine residues. In bovine airway smooth muscle, ERK activation is induced by a wide variety of stimuli, including PDGF, EGF, IGF-1, 5-HT [50, 74], bradykinin [75], α-thrombin [75, 76], ET-1 [56, 76] and hydrogen peroxide [77]. Several independent groups have reported that the duration of ERK activation is critical for transduction of the mitogenic stimulus. KELLEHER *et al.* [74] and MALARKEY *et al.* [75] observed that, in bovine airway smooth muscle cells, ERK activation was of significantly longer duration following stimulation with mitogens (PDGF and ET-1) than following treatment with nonmitogens (bradykinin). A similar finding of sustained ERK activation by PDGF was reported in guinea-pig airway smooth muscle cells by PYNE *et al.* [78]; whereas ERK activation by bradykinin was again more transient. Data that further support the requirement

for ERK activation in the proliferation of cultured airway smooth muscle cells are derived from inhibitor-based studies [56, 75, 79, 80]. PD98059, an inhibitor of MEK-1 activation, which is required and sufficient for ERK activation, has been shown to inhibit both PDGF-stimulated activation of ERK and DNA synthesis [80]. However, at concentrations of PD98059 which abolished PDGF- and α-thrombin-stimulated $p42^{ERK2}$ activation, DNA synthesis was only partially inhibited [81], suggesting that ERK activation is necessary, but not sufficient, for a full mitogenic response and raises the possibility that ERK-independent pathways (discussed later) are also involved and important in the proliferation of airway smooth muscle.

Studies which have focused on the upstream regulators of ERK and MEK-1 have demonstrated in both rat and bovine cultured tracheal smooth muscle cells that Raf-1 is catalytically activated by PDGF [79, 82] and hydrogen peroxide [79]. Furthermore, activation of MEK-1, but not MEK-2, activity occurred following stimulation with hydrogen peroxide [79], and was reduced by the MEK inhibitor, PD98059. Interestingly, S-phase entry and proliferation, as determined by BrdU labelling or ^{3}H-thymidine incorporation, could not be induced in these cells by hydrogen peroxide [79]. This may reflect a requirement for sustained ERK activation in proliferation [74, 75, 78], or that activation of the p38MAPK or SAPK/JNK pathway by hydrogen peroxide in these cells [79] may in some way counter the effect of ERK activation.

Alternative pathways of extracellular signal-regulated kinase activation by polypeptide growth factors in airway smooth muscle

As discussed above, the classical pathway for activation of ERK in airway smooth muscle and other cells appears to require the sequential activation of Ras, Raf-1 and MEK-1. However, HERSHENSON *et al.* [50] have provided evidence in bovine airway smooth muscle that ERK activation by polypeptide growth factors may occur independently of the Raf-1 intermediary. In support of this, activation of adenylyl cyclase activity by pretreatment with forskolin significantly reduced both EGF- and PDGF-stimulated activation of Raf-1, but had no effect on ERK activity [50, 76]. Since these [50, 76] and other groups [80] have demonstrated that MEK-1 is necessary and sufficient for ERK activity, other pathways that are independent of Raf-1 must exist, representing alternative routes to MEK-1 activation.

A recent series of articles has characterized the existence in airway smooth muscle of some of these possible alternative pathways. PAGE *et al.* [83], using expression of a dominant-negative form of H-Ras, have shown that, activation of ERK by PDGF is critically dependent on Ras unlike Raf-1. Furthermore, in the same study, expression of a constitutively active Ras induced not only activation of ERK and JNK1 but also transcriptional activation of the downstream cyclin D_1 promoter. Microinjection of airway smooth muscle cells with a neutralizing antibody directed against the product of the transcriptional activation of this promoter, cyclin D_1, has demonstrated its importance in S-phase traversal [84]. Ras-dependent activation of JNK1 and activation of the JNK/SAPK and p38 pathways was also reported by PAGE *et al.* [83], but was found to be modest and insufficient for activation of the cyclin D_1 promoter, suggesting that in airway smooth muscle this pathway is not involved in cell cycle progression when activated by Ras alone [83]. Further investigation, however, revealed that the critical pathway in this process was MEK-1-dependent, since induction of transcriptional activation of the cyclin D_1 promoter by the introduction of constitutively active MEK-1 was blocked by the MEK-1 inhibitor, PD98059 [83]. More recently, the critical importance of Ras proteins in cell cycle traversal into the S phase was confirmed in a study in which the fractional labelling of BrdU in human airway smooth muscle cells, stimulated with EGF, was reduced following microinjection of an anti pan-Ras neutralising antibody [85]. These findings consolidate previous reports by these and other groups that ERK activation in airway smooth muscle regulates the transcriptional activation of the cyclin D_1 promoter, and that Ras proteins, MEK-1 and ERK activation probably constitute a discrete pathway to cyclin D_1 expression in these cells (see fig. 2) [83, 86]. However, as pointed out in a recent review by HERSHENSON and ABE [87], activation of ERK and induction of cyclin D_1 expression alone may not be sufficient for cell cycle entry. In other cell systems additional key events are required for transition from the G_1 to the S phase. These include the binding of cyclin D_1 to cyclin-dependent kinase (cdk)4 to form a complex which, following its activation by cyclin-activating kinase, phosphorylates the retinoblastoma protein (pRb) and reduces its affinity for the E2F nuclear transcription factor. This allows derepression of E2F and transcriptional activation of specific S phase-dependent genes. Other key events include induction of cyclin A expression and degradation of the cdk inhibitor, $p27^{Kip1}$. Thus the apparently paradoxical observation that Ras proteins are critically important for cell cycle traversal into the S phase [85], and that ERK activation of cyclin D_1 expression is necessary [83, 84, 86] but not sufficient for a full mitogenic response [81], reiterates the possibility that Ras proteins can co-ordinate S phase entry by regulating cell signalling through both ERK-dependent and ERK-independent pathways (discussed later).

In addition to Ras, other Raf-1-independent MEK-1 activators have been sought in airway smooth muscle, but with little success. In other cell systems these include additional serine/threonine protein kinase members of the *raf* family, such as A-Raf, B-Raf, MEK-1 kinase (MEKK-1), Tpl-2 and Mos. Using lysates of PDGF-stimulated bovine cultured tracheal smooth muscle cells, treated with forskolin to block the activity of Raf-1, dual function kinases that phosphorylate ERKs have been identified in fractions resolved by anion-exchange chromatography [73]. However, when these fractions were probed with antibodies directed against known MEK-1 activators (*i.e.* A-Raf, B-Raf, MEKK-1, Tpl-2 and Mos), none could be detected, suggesting the presence of a novel PDGF-stimulated forskolin-insensitive MEK-1 kinase. Although this study awaits confirmation, it raises the possibility that other novel PDGF-stimulated kinases which account for Ras-dependent Raf-1-independent ERK activation in airway smooth muscle remain to be identified.

Extracellular signal-regulated kinase activation by G protein-coupled receptors

The above discussion explains, in part, ERK-dependent airway smooth muscle cell proliferation induced by polypeptide growth factors acting through RTK-linked receptors. The upstream signalling events involved in ERK-dependent proliferation by G protein-coupled receptor agonists are now addressed. Although not easily explained at first, it was known that the modest proliferation of airway smooth muscle in response to ET-1 was mediated by G protein-coupled ET_A [55] and ET_B receptors [56, 82] (depending on the species investigated), and that this was dependent upon activation of the ERK pathway. Likewise, proliferation induced by 5-HT was also shown to be dependent on ERK activation [50, 74]. In other cell systems, several studies have demonstrated that G protein-coupled receptors (*e.g.* α-thrombin) activate ERKs and stimulate cell growth *via* a pertussis toxin-sensitive mechanism involving direct nucleotide exchange on the small molecular weight G protein, p21 Ras [88, 89]. Similarly, MALARKEY *et al.* [75] demonstrated, in bovine cultured tracheal smooth muscle, that ERK activation induced by ET-1, but not PDGF, was mediated through a pertussis toxin-sensitive pathway that was predominantly PKC-independent (see next section for discussion). SHAPIRO *et al.* [76] showed that inhibiting the kinase activity of Raf-1 with forskolin prevented ERK activation by ET-1, but not by PDGF, suggesting that unlike RTK-linked receptors, G protein-coupled receptors have a requirement for Raf-1 in ERK activation. A similar requirement for Raf-1 in ERK activation was also found for 5-HT, contrasting with ERK activation by PDGF which was again found to be independent of Raf-1 [50]. In a later study, the requirement for Raf activation by G protein-coupled receptors in ERK activation was redetermined by reconstitution of the pathway in Chinese hamster ovary (CHO) cells by means of cotransfection with plasmids expressing ETB and ERK, as well as various active and kinase-dead Raf mutants [82]. The data confirmed that Raf-1 was a critical upstream Ras-dependent regulator of ERK in the ET signalling pathway [82]. Consistent with this model, PANETTIERI and coworkers [85] showed that stimulation of DNA synthesis by α-thrombin or EGF in human airway smooth muscle cells could be prevented following microinjection of an antipan-Ras neutralizing antibody, suggesting that Ras proteins are critically important for induction of growth by not only RTK-linked polypeptide growth factors such as EGF but also G protein-coupled receptor agonists such as α-thrombin.

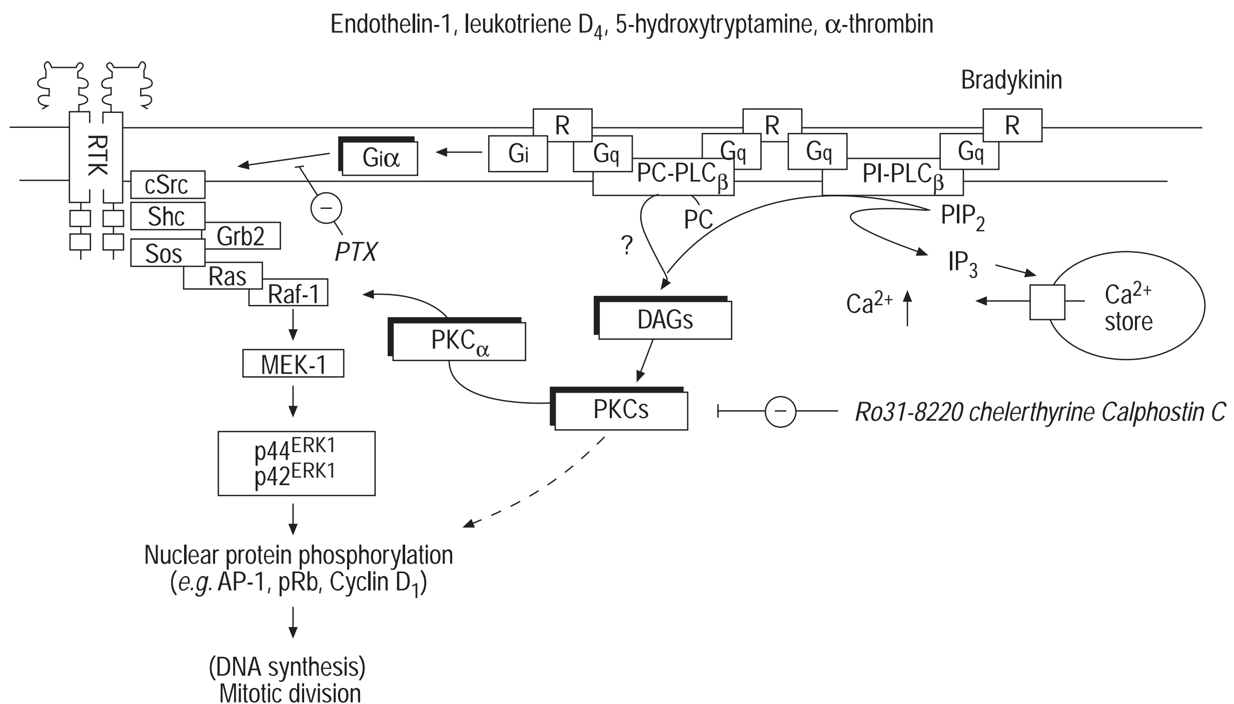

Fig. 3. – Possible mechanisms of extracellular signal-regulated kinase (ERK) activation by agonists that couple pertussis toxin (PTX)-sensitive G protein-linked receptors (R) in airway smooth muscle. Evidence in airway smooth muscle suggests ERK activation by inhibitory G protein (Gi)-coupled, but not protein tyrosine kinase (RTK)-linked, receptors occurs by means of a PTX-sensitive Raf-1-dependent pathway [94]. In contrast to other cell systems, this appears to be mediated by Giα subunits associating with Ras. (See fig. 4 for role of Giβγ subunits.) Agonists such as bradykinin, acting exclusively through Gq-coupled receptors, mobilize intracellular calcium and activate protein kinase C (PKC), but do not activate Ras or induce proliferation. Activation of PKC is also implicated in airway smooth muscle mitogenesis, although the importance of phosphatidylinositol (PI)- or phosphatidylcholine (PC)-dependent phospholipase C (PLC)-derived diacylglycerols (DAGs) is disputed. Currently, no clear consensus for the site of integration of PKC activation of ERK in airway smooth muscle has been established, although in other systems, PKCα may phosphorylate and activate Raf-1 [96]. The dotted arrow illustrates possible direct effects of PKC on the regulatory elements of activation protein 1 (AP-1). Pharmacological inhibitors are shown in italics. Shc: SH-domain-containing protein; Grb2: guanine nucleotide-releasing-binding protein 2; MEK-1: mitogen-activated protein kinase/ERK-activating kinase 1; $p44^{ERK1}$ and $p42^{ERK2}$: ERK isoforms; pRb: retinoblastoma protein; PIP_2: phosphatidylinositol 4,5-biphosphate; IP_3: inositol 1,4,5-triphosphate; DNA: deoxyribonucleic acid.

In the study of Posada and coworkers [82], it was also demonstrated that Raf-1 activity and the subsequent activation of ERK by ET also required the influx of calcium that was mediated by a dihydropyridine-sensitive pathway. Calcium influx alone, however, was not sufficient to activate ERK or indeed cell proliferation, consistent with previous observations by Panettieri *et al.* [58] who investigated other G protein-linked receptor agonists, and showed that, although α-thrombin was capable of mobilizing calcium and stimulating proliferation, mobilization of calcium by bradykinin failed to induce proliferation. However, as discussed earlier, this may involve differences in the capacity of each agonist to promote sustained ERK activation in airway smooth muscle, and in the ability of their receptors to couple to both G_i and G_q. Although prevention of calcium influx was reported to prevent Raf-1 activity in airway smooth muscle cells, Raf-1 itself is not thought to be calcium-dependent [82]. The possibility arises, therefore, that additional elements with a requirement for calcium lie upstream of Raf-1 activation following stimulation by ET in airway smooth muscle.

Although no clear consensus has yet emerged in airway smooth muscle, there appear to be a number of potential routes whereby G protein-coupled receptors can activate ERKs. In other cell systems, depending on the receptor that is activated, ERK activation can be mediated by pertussis toxin-sensitive (*i.e.* G_i or G_o) [90, 91] or pertussis toxin-insensitive (*i.e.* G_q) [92] G-protein-coupled pathways, which are dependent on the activation of either PKC [50, 92] or Ras proteins [90, 91, 93]. Emala *et al.* [94], confirming the observations of others [75, 82], recently showed in human cultured airway smooth muscle cells that activation of Ras by ET was blocked by pertussis toxin, suggesting that the receptors for ET, which activate Ras, couple G_i but not G_q. Furthermore, in the same study, overexpression of a G protein βγ-subunit scavenger failed to prevent activation of Ras, indicating that ET receptors most probably couple to a G_i that activates Ras *via* the $G_i\alpha$ subunit [94] and not a $G_i\beta\gamma$ (or $G_q\beta\gamma$) subunit as proposed in other cell types [92, 95] (fig. 3). Bradykinin, which does not stimulate proliferation of human airway smooth muscle cells, and histamine, which is poorly mitogenic, failed to activate Ras [85, 94], and instead were shown to couple to G_q [94], supporting the model that activation of G_i- but not G_q-coupled receptors in airway smooth muscle leads to activation of Ras by ET-1. Thus, ERK activation induced by agonists that stimulate receptors coupled to G_q (linked to calcium mobilization) but not G_i, such as bradykinin and histamine, occurs *via* a pathway that is independent of Ras. Agonists, such as ET and α-thrombin, which also mobilize intracellular calcium *via* G_q, must therefore require an additional input from G_i for activation of Ras and induction of DNA synthesis (fig. 3). Further studies are required, but it seems likely that this is the basis for the altered kinetics of ERK activation and lack of mitogenic efficacy observed with bradykinin and histamine, compared to ET and α-thrombin [85, 94].

Surprisingly, activation of ERK in guinea-pig airway smooth muscle by cSrc, an upstream nonreceptor tyrosine kinase which in other cell systems functions as an intermediate between $G_i\beta\gamma$ and Ras-dependent ERK activation, was reduced by 50% with pertussis toxin [100]. These preliminary data suggest that, in addition to directly associating with and activating cSrc *via* a pertussis toxin-insensitive mechanism, the PDGF-RTK can utilize the pertussis toxin-sensitive G-protein, G_i, to regulate cSrc, perhaps by generation of free $G_i\beta\gamma$, to achieve a more efficient or greater stimulation of the ERK cascade. This may also offer some explanation for the synergy observed between PDGF and agonists which interact with G_i-coupled receptors [55].

Alternative pathways for proliferation: the protein kinase C pathway

PKC is the largest known family of the serine/threonine-specific kinase family. Once thought to be a single enzyme, PKC is now known to comprise a large family of enzymes with varying structure, cofactor requirements and function [101]. Its abundance and variation in both tissue and cellular distribution probably accounts for its participation in a vast array of signal transduction functions. Classically, hydrolysis of membrane phosphoinositides, particularly phosphatidylinositol 4,5-biphosphate (PIP_2) to 1,2-*sn* diacylglycerol (DAG), and the subsequent activation of PKC in airway smooth muscle is mediated by phosphatidylinositol (PI)-dependent phospholipase C (PLC) coupled to G_q-linked receptors (see fig. 3) [101]. Under these conditions, however, the generation of PIP_2 in the membrane and activation of PKC is only transient [101], suggesting that activation of this pathway by bronchoconstrictor agonists, such as acetylcholine, histamine, 5-HT, bradykinin and α-thrombin, is important only for shorter-term events typified by the development of contractile tone. Recent evidence suggests that agonist-induced hydrolysis of another membrane phospholipid, phosphatidylcholine (PC), by PC-dependent PLC produces a prolonged increase in DAG levels, which has been hypothesized to underlie the sustained activation of PKC and to participate in longer-term events such as the maintenance of contractile force and potentially the induction of proliferation by contractile agonists (see [101, 102] for reviews). Another PLC isoform, PLCγ, contains SH2 and SH3 src-homology domains and is linked to RTK activation and has been proposed to be involved in cellular proliferation.

Although the contribution of PI and PC hydrolysis to DAG production has not been resolved in airway smooth muscle, a possible role for PKC in airway smooth muscle mitogenesis was first suggested by Panettieri *et al.* [103] who demonstrated that prolonged stimulation of human airway smooth muscle cells with phorbol esters induced DNA synthesis and expression of *c-fos*. Few subsequent studies have characterized the effect of direct stimulation of PKC using phorbol esters. Instead, many investigators have sought to examine more physiological cell surface receptor-mediated stimuli in airway smooth muscle cells, in which endogenous DAG levels have been manipulated or PKC depletion strategies based on prolonged pretreatment with phorbol esters employed [77, 79, 104, 105]. In canine airway smooth muscle, PKC downregulation markedly attenuates phorbol ester-induced phosphorylation of a 76-kDa protein (presumably autophosphorylated PKC) and abolishes both the proliferative response and expression of *c-fos* [106–108]. In bovine tracheal smooth muscle, stimulation of proliferation by β-hexosaminidases (mannosyl-rich lysosomal hydrolases),

at mannose-recognizing receptors, acting was enhanced by increasing endogenous DAG levels using the DAG kinase inhibitor, R59022 [104]. Likewise depletion of PKC by prolonged treatment with phorbol ester reduced the proliferative response [104]. It has also been shown that prolonged pretreatment of airway smooth muscle cells with the phorbol ester, 4β-phorbol myristate acetate, but not the inactive 4α-isomer, markedly attenuated the subsequent proliferative response induced by serum [105] or PDGF-BB [109].

To support the view that activation of PKC is a necessary event for proliferation of airway smooth muscle, investigators have also examined the effect of selective inhibitors of PKC activity. LEW *et al.* [104] found that pretreatment of bovine tracheal smooth muscle cells with calphostin C prevented proliferation following activation of mannose-recognizing receptors by β-hexosaminidases. Proliferation of rabbit tracheal smooth muscle cells induced by serum was also inhibited by the bisindolylmaleimides, Ro31-8220 and its less potent analogue Ro31-7549 [105]. In the same study, these inhibitors also abolished Ca^{2+}-dependent and -independent PKC activity purified from airway smooth muscle cells. In other studies these PKC inhibitors have been found to be effective in preventing proliferation of human [109] and rabbit [110] airway smooth muscle induced by PDGF isoforms acting through distinct PDGF receptors. These findings contrast with those of others in bovine tracheal smooth muscle cells, in which calphostin C [104] and other selective PKC inhibitors such as GF109203X, Ro31-8220 and chelerythrine [81] failed to inhibit PDGF-stimulated DNA synthesis, but are in agreement with another report in human airway smooth muscle in which proliferation, induced by PDGF-BB, was dependent not only on tyrosine kinase-dependent mechanisms but also on PLC-dependent processes, in which PKC activation is believed to be a downstream event [111].

Expression and function of protein kinase C isoforms in proliferation

Similar patterns of expression of PKC isoforms have been found in airway smooth muscle from the large central airways of several species. Information, however, is not available regarding the expression of PKC isoforms in the smooth muscle from smaller airways, the pathophysiologically more relevant site of smooth muscle remodelling. In fresh contractile bovine trachealis, there is expression of the conventional α-, βI- and βII-PKC isoforms as well as the novel δ-, ε- and θ-PKC isoforms and also trace amounts of the atypical ζ-isoform [112]. Canine trachealis shows a similar profile, except that PKCα was not detected [113]. With the availability of monoclonal antibodies to additional members of the atypical PKCs, a more recent study in porcine trachealis has also detected expression of the PKCμ and -ι/λ isoforms [114]. In fresh human trachealis there is expression of conventional PKC isoforms (α, βI, βII) as well as novel (δ, ε, ν, θ) and atypical (ζ) variants [115]. In cases in which discrepancies between species have been noted in the expression of particular isoforms, it is likely that this involves failure of antibodies raised against human epitopes to cross-react with those of different species, or the presence of contaminating nonmuscle cell types in the tissue preparations. PKCγ, often abundantly expressed in neural tissues, has not been detected in airway smooth muscle from any species so far investigated.

Little information is available regarding the functions of specific PKC isoforms in smooth muscle, in particular regarding which isoforms are involved in regulating the growth status or state of differentiation of airway smooth muscle. ASSENDER *et al.* [116] reported that nonproliferating human and rat vascular smooth muscle cells showed a similar qualitative pattern of PKC isoform expression (α-, δ- and ζ-PKC) to proliferating smooth muscle cells in culture. Analysis of the messenger ribonucleic acid (mRNA), however, revealed that, unlike their proliferating counterparts, quiescent smooth muscle cells did not express mRNA for PKCε. Thus, although PKCα was expressed in smooth muscle cells in all states of differentiation, expression of PKCε was increased as smooth muscle cells became more proliferative. Expression of PKCβ was not detected in either freshly isolated or proliferating smooth muscle cells. In another study, PKCα was downregulated when rat vascular smooth muscle cells assumed a proliferative/secretory phenotype [117]. When the cells reassumed their contractile state, PKCα expression reverted to normal levels.

Phenotype-dependent expression of PKC isoforms may occur in airway smooth muscle. In canine tracheal smooth muscle cells, HALAYKO *et al.* [39], using an antibody which recognizes the α- and β-PKC isoforms (note that PKCα was reported not to be expressed in canine trachealis [113]), reported that expression of PKCβ was increased by eight-to 10-fold in proliferating compared to contractile cells. This is consistent with the authors′ unpublished observations in human airway smooth muscle, in which increased expression of PKCβ, as well as PKCε, was found in proliferating cells, compared to their serum-deprived nonproliferating counterparts (S.J. Hirst, unpublished observations). At this stage, however, it is uncertain whether such changes precede or follow cell division, or whether they are necessary or sufficient for altered smooth muscle function.

Since the function of the various PKC isoforms expressed in airway smooth muscle has not been investigated in any detail, other tissues must be looked to for possible clues. In nonmuscle cell types, the PKC βII- and ζ-isoenzymes are reported to be essential for differentiation and proliferation [118, 119]. In human arterial smooth muscle cells, PKCα activation is necessary for progression from the G1 to S phase of the cell cycle and subsequent proliferation [120], and selective depletion of PKCα by antisense strategies prevents proliferation of rat arterial smooth muscle cells [121]. In contrast, PKCδ, a Ca^{2+}-independent PKC isoform also expressed in airway smooth muscle, has previously been shown to retard cell cycle progression in G_1 through an effect on cell cyclins [122], suggesting that activation of different PKC isoforms in smooth muscle may lead either to growth acceleration or inhibition. ASSENDER *et al.* [116], using Western immunoblot analyses, reported that PKCζ expression was present in both proliferating and contractile rat vascular smooth muscle cells. A study examining PKCζ activity in chicken gizzard smooth muscle suggests that this isoform differs very markedly from other PKC isoforms in its susceptibility to downregulation by phorbol esters and in

its inhibition by PKC inhibitors such as sphingosine, chelerythrine, staurosporine or calphostin C [123]. Given that airway smooth muscle proliferation occurring in response to a range of stimuli can be prevented by prolonged pre-exposure of cells to phorbol esters or the presence of inhibitors of PKC, it might seem appropriate to conclude that, at least in airway smooth muscle this PKC isoform is not involved directly in the proliferative process. However, a recent preliminary report has been presented indicating that transfection of human airway smooth muscle cells with a dominant negative PKCζ inhibits PDGF-stimulated DNA synthesis by as much as 80% [124]. In a follow-up study, and in contrast to the authors′ unpublished findings, it was demonstrated that proliferation was associated with upregulation of PKCζ which occurred without any change in the expression of other PKC isoforms [125].

Although there seems little agreement regarding the patterns of expression of particular PKC isoforms in contractile or proliferative vascular or airway smooth muscle cells, the ability of Ro31-8220 and Ro31-7549 to abolish global Ca^{2+}-dependent and -independent PKC activity in airway smooth muscle, and, in the same study, to inhibit proliferation [104], is consistent with the expression and function of at least one or more of these isoenzymes in the proliferative process. Furthermore, both Ro31-8220 and Ro31-7549 have been reported to potently and selectively inhibit α-, βI-, βII- and ε-PKC isoenzymes from other cell systems [126], and these same isoforms are expressed in airway smooth muscle obtained from several species including the human.

Interaction of protein kinase C with other signalling molecules in proliferation

Although most investigators now agree that activation of PKC is an important factor in the cascade of biochemical events that lead to proliferation of airway smooth muscle (particularly in response to G protein-coupled receptor activation), details of the relevant downstream events that trigger cell division are only just emerging. WHELCHEL *et al.* [56] demonstrated, in cultured rat tracheal smooth muscle, that activation of the ERK pathway by ET-1, was prevented following phorbol ester-induced PKC depletion, emphasizing a requirement for PKC. Similar results have also been reported using the PKC inhibitor, Ro31-8220, which partially reduced ET-1-stimulated MAPK activation in bovine tracheal smooth muscle [75]. ABE *et al.* [79] had previously demonstrated a similar requirement for PKC in $p42^{ERK2}$ activation in airway smooth muscle following stimulation with hydrogen peroxide. However, this requirement for PKC in $p42^{ERK2}$ activation is not universally observed following activation of G protein-coupled receptors, since α-thrombin is reported to activate ERK even in PKC-depleted cells. However, in other smooth muscles, α-thrombin upregulates expression of PDGF-A chain ligands and their corresponding α-receptors [127]. Like PDGF-β receptors, PDGF-α receptors have intrinsic tyrosine kinase activity and retain the ability to activate $p42^{ERK2}$ following PKC depletion [56]. Concomitant upregulation of PDGF ligands and receptors by α-thrombin may also help explain the potent and efficacious mitogenic activity of this proteinase in airway smooth muscle.

Of significance in reconciling the above data supporting the interaction of PKC- and ERK-dependent mitogenic signalling events in airway smooth muscle is that inhibition of MEK-1 by PD98059 prevents both ET-1- and PDGF-stimulated ERK activation and cell proliferation. This might underscore the need for both PKC- and ERK-dependent mechanisms to operate in airway smooth muscle for transduction of the mitogenic signal and suggests that the locus of the interaction of PKC with $p42^{ERK2}$ is upstream of MEK-1 [56]. One possible mechanism for integration of the PKC signal into the MAPK cascade, which is consistent with its activation by G protein-coupled receptors [76], is that PKC is required to activate Raf-1. In addition to tyrosine kinase-dependent phosphorylation of Raf-1 by polypeptide growth factors, direct phosphorylation by PKCα is reported to stimulate the catalytic activity of Raf-1 [96]. In support of this, direct activators of PKC (phorbol esters) [82], as well as agonists which utilize the PKC pathway such as ET-1 [76] and 5-HT [50], activate Raf-1 kinase activity in airway smooth muscle cells. Furthermore, hydrogen peroxide is reported to promote ERK activation in airway smooth muscle by successive activation of PKC, Raf-1 and MEK-1 [79]. Finally, LEW *et al.* [104] have demonstrated that induction of proliferation in bovine tracheal smooth muscle by β-hexosaminidase and activation of the ERK pathway is prevented by the PKC inhibitor calphostin C, an effect which appears to be mediated at the Ras/Raf-1 locus (fig. 3) [128].

Alternative pathways for proliferation: the phosphoinositide 3-kinase pathway

PI3K was originally characterized as a phosphoinositide kinase activity associated with antiphosphotyrosine immunoprecipitates in PDGF-stimulated fibroblasts [129]. Under *in vitro* conditions, this lipid kinase activity was found to phosphorylate PI, PI 4-phosphate (PIP) and PIP_2 at the 3-position of the inositol ring to generate the phosphoinositides, PIP, PIP_2 and PI 3,4,5-triphosphate (PIP_3). The preferred substrate *in vivo*, however, appeared to be the minor lipid constituent of the plasma membrane namely PIP_2 [130], also a major substrate for PLC. PI3K was subsequently characterized as a heterodimer consisting of a p85 regulatory/adaptor subunit and a p110 catalytic subunit.

Isoforms of phosphoinositide 3-kinase

A large number of PI3K isoforms have now been identified and these have been classified into three groups according to their structural properties and substrate specificities [131]. Class I PI3K, which represent the major subgroup, are activated following ligand binding to a receptor at the cell surface and utilize PIP_2 as the preferred substrate. Two subclasses have also been described. Class Ia PI3K is activated by growth factor receptors and comprises a p110α or β-catalytic subunit and a p85 regulatory subunit containing two SH2 domains; class Ib PI3K (also known as PI3Kγ), initially identified in neutrophils and

platelets, comprises a G protein Gβγ-regulated p101 adaptor subunit and a p110γ catalytic subunit and is activated by βγ subunits derived from heterotrimeric G protein-coupled receptors [132]. Common to all class I PI3K p110 catalytic subunits is a phosphoinositide kinase domain, a protein kinase domain and a Ras-binding domain with a p85 binding domain also present in the case of class Ia enzymes [131, 133]. Class II PI3K consist of a catalytic subunit only, and utilize PI, PIP and PIP_2 as substrates. Class III PI3K, originally described in yeast, appear to only utilize PI as a substrate. The catalytic subunit is composed of a lipid kinase domain and a serine/threonine protein kinase domain. The catalytic subunit of the human form of this PI-specific (class III) PI3K binds to a 150-kDa binding/regulatory subunit termed p150 and has been postulated to play a role in the regulation of vesicle trafficking similar to that observed in yeast [134].

Activation of phosphoinositide 3-kinase

Activation of PI3K class I enzymes, the predominant form identified in smooth muscle, can be achieved by a number of mechanisms (fig. 4). The classical pathway involves activation of polypeptide growth factor receptors such as PDGF, which induces autophosphorylation of specific tyrosine residues, allowing these receptors to interact with the SH2 domain of the p85 regulatory adaptor subunit of PI3K, resulting in its localization to the membrane and activation of the kinase activity [135]. Other growth factor receptors act to phosphorylate an intermediate adaptor protein on tyrosine residues that binds p85 through its SH2 domain and induces an increase in PI3K specific activity; an example of this latter mechanism is the IGF-1 receptor, which phosphorylates IRS-1, an adaptor protein, which then recruits PI3K [136]. Direct interaction of PI3K following ligand binding with nonreceptor tyrosine kinases such as $pp60^{cSrc}$ is also known to occur, with the SH3 domains of these src-family kinases interacting with two proline-rich regions on p85 to induce activation of the enzyme [137]. In other cell types, the ability of G protein-coupled receptors to activate the p101/p110γ form of PI3K (class Ib or PI3Kg) is known to involve βγ subunits. Interestingly, overexpression of PI3Kγ in COS-7 cells resulted in MAPK activation in a βγ-dependent manner and expression of a mutated enzyme lacking lipid kinase activity abolished these responses [138]. This study also noted that stimulation of MAPK by PI3Kγ required a tyrosine kinase which phosphorylated the adaptor Shc, causing its association with Grb2 and activation of Ras. However, a separate study, which examined the effects of a "lipid

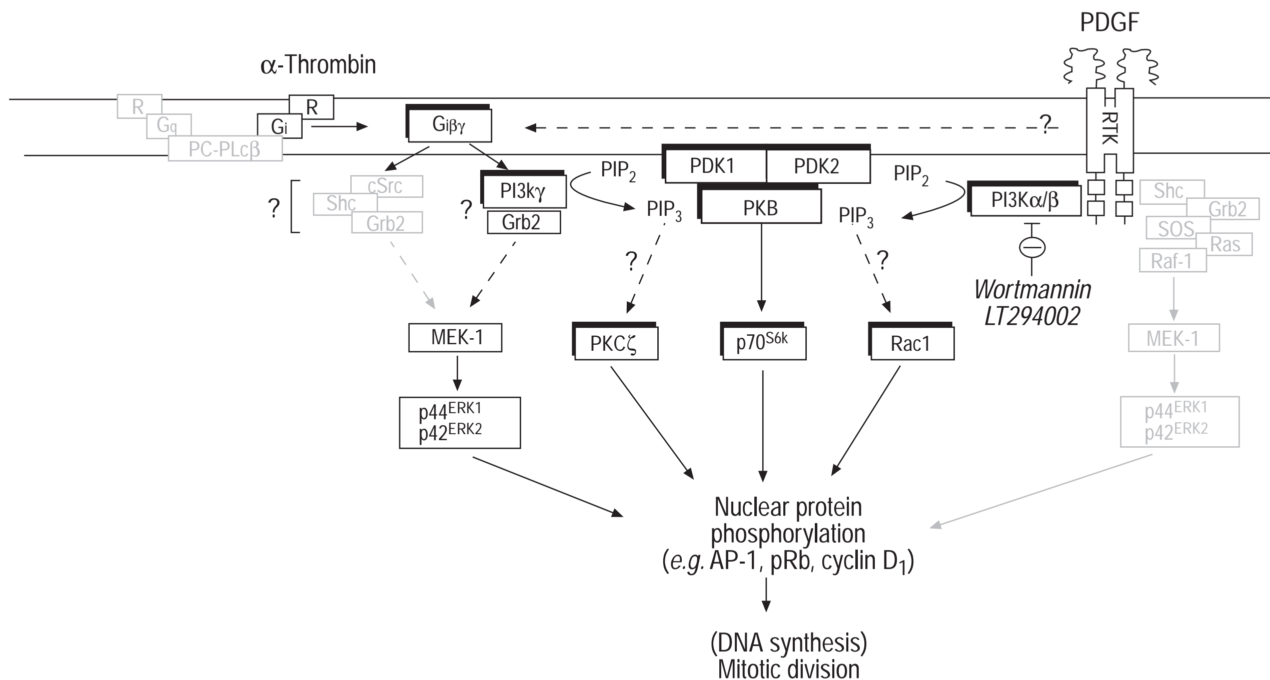

Fig. 4. – Proposed mechanisms for activation of the phosphoinositide 3-kinase (PI3K) pathway in airway smooth muscle. Activation of inhibitory G protein (Gi)-coupled receptors (R) by agonists such as α-thrombin may activate the γ-isoform of PI3K by direct association with Giβγ subunits and guanine nucleotide-releasing-binding protein 2 (Grb2). Grb2 may be a site at which PI3K is integrated into the extracellular signal-regulated kinase (ERK) cascade (shown in grey) with PI3Kγ acting as an adaptor protein [97]. Alternatively, activation of protein tyrosine kinase (RTK)-linked receptors by polypeptide growth factors such as platelet-derived growth factor (PDGF) may recruit PI3Kα/β isoforms following autophosphorylation of key tyrosine residues. PI3K activation by other growth factors such as epidermal growth factor (EGF) may involve transactivation of ErbB2 by the EGF receptor (not shown) [98]. Localized generation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) by PI3K recruits protein kinase B (PKB) to the plasma membrane, where it can be fully activated by dual phosphorylation by the phosphoinositide-dependent kinases (PDK)-1 and -2. Dual phosphorylated PKB then activates the S6 ribosomal kinase ($p70^{S6k}$), which is linked to cyclin D_1 expression in these cells (see text for details). PIP_3 may also activate Rac1 as well as activating PKCζ and this may offer another integration site for PKC in airway smooth muscle mitogenesis. Although Ras is not shown, there is evidence in other cell systems that the p110 catalytic subunit of PI3K can be activated by direct interaction with Ras [99]. The action of pharmacological inhibitors at PI3Kα/β and PI3Kγ (omitted for clarity) is shown in italics. PC: phosphatidylcholine; PLC: phospholipase C; Shc: an SH-domain-containing protein; MEK-1: mitogen-activated protein kinase/ERK-activation of kinase 1; $p44^{ERK1}$ and $p42^{ERK2}$: ERK isoforms; PIP_2: phosphatidylinositol 4,5-biphosphate; AP-1: activation protein 1; pRb: retinoblastoma protein; DNA: deoxyribonucleic acid.

kinase-dead" form of PI3Kγ in mediating lysophosphatidic acid-induced MAPK activation, found this to be as effective as wild-type PI3Kγ [139]. These observations suggest a role for PI3K as not only a lipid and protein kinase but also a potential adaptor protein in the activation of MAPK (fig. 4). A further important upstream regulator of PI3K, also implicated in activation of the MAPK pathway, is Ras, which has been postulated to interact directly with the p110γ catalytic subunit and induce activation [99], however, the mechanism and consequence of this interaction remains to be fully elucidated.

The development of the selective inhibitors of PI3K, wortmannin and LY294002, has greatly facilitated the search for downstream targets of PI3K and, in particular, has allowed dissection of the importance of this pathway in mediating agonist-stimulated proliferation of airway smooth muscle. Potential targets of PIP_3, generated as a result of PI3K activation, include $p70^{S6k}$, protein kinase B (also known as Akt or Rac protein kinase and related to PKC), novel PKCs, PLCγ, Rho family GTPases (Rho A-C, Rac1 and Rac2, and cdc42) and other low-molecular-weight signalling intermediates such as Vav, Tiam1, ARNO, centaurin-α and DAPP1. In various cell types, mitogenic stimuli induce activation of $p70^{S6k}$, resulting in phosphorylation of the 40-S ribosomal protein S6, an important event in G_1 cell cycle transition. Selective inhibitors of PI3K prevent activation of $p70^{S6k}$, and transfection of constitutively activated PI3K results in phosphorylation and activation of $p70^{S6k}$ [140].

The first direct evidence demonstrating the possible importance of this pathway in proliferation of airway smooth muscle was provided by Scott *et al.* [141]. Their study demonstrated that induction of DNA synthesis in bovine airway smooth muscle by PDGF occurred in a PI3K-dependent manner which involved the activation of $p70^{S6k}$ (fig. 4). Activation of protein kinase B, itself a serine/threonine protein kinase, was also shown to be inhibited by wortmannin following receptor activation of airway smooth muscle, indicating a similar dependency on PI3K activation [141]. In other cell systems, further investigation has now detailed the mechanism by which PI3K mediates activation of protein kinase B. Localized generation of PIP_3 following receptor-stimulated PI3K activation recruits protein kinase B to the plasma membrane, where it undergoes phosphorylation at threonine^{308} and serine^{473} by phosphoinositide-dependent kinase (PDK)-1 and a putative PDK2, which are PIP_3-dependent protein kinases also containing PH domains. This dual phosphorylation reaction results in full activation of protein kinase B [142]. Of interest, PDK1 can also phosphorylate and activate PKCζ and -δ and $p70^{S6k}$, indicating the possibility of a number of interlinked signalling pathways [143]. A recent report has demonstrated that protein kinase B is activated in airway smooth muscle by the mitogens PDGF and α-thrombin in a PI3K-dependent manner, and this effector presumably lies upstream of $p70^{S6k}$ on the pathway which appears to drive cellular proliferation (fig. 4) [81].

Novel (ε, ν) and atypical (ζ and λ) isoforms of PKC have been shown to be regulated by PI3K. These PKCs contain incomplete DAG-binding domains and it has been proposed that these may mediate interaction with PIP_3 [144]. Thus activation of PI3K in the mitogenic signalling pathways of airway smooth muscle may explain the marked reduction in PDGF-stimulated DNA synthesis reported in human airway smooth muscle cells transfected with a dominant negative mutant PKCζ [124, 125]. Activation of PI3K has also been shown to be required for Rac guanosine diphosphate/GTP exchange [145], and inhibition of PI3K prevents the ability of Rac to activate the JNK/SAPK cascade which phosphorylates c-Jun [146]. Recently, it has been proposed that activation of PLCγ may occur upon binding of PIP_3 with either the PH domain or the SH2 domains of the lipase.

Role of phosphoinositide 3-kinase activation in proliferation

Few investigators have studied the role of PI3K activation in modulating proliferation of airway smooth muscle. Scott *et al.* [141] demonstrated a critical role for PI3K-dependent $p70^{S6k}$ activation following induction of DNA synthesis by PDGF in bovine tracheal smooth muscle cells. Importantly, a previous study by this group had shown that the mitogenic potential of an agonist was unrelated to the initial magnitude of ERK activation [75]. An essential requirement for PI3K in mediating EGF- and α-thrombin-induced proliferation of human airway smooth muscle has also been reported by Krymskaya *et al.* [147]. Their report demonstrated that expression of a constitutively active p110 resulted in activation of $p70^{S6k}$, and expression of a dominant negative mutant p85 blocked mitogen-induced $p70^{S6k}$ activation and DNA synthesis. Furthermore, Walker *et al.* [81] had previously shown that PDGF and α-thrombin induce a rapid activation of PI3K and PIP_3 generation, and that the magnitude of both enzyme activation and PIP_3 accumulation correlates closely with the mitogenic potency of these two agonists. This study also re-evaluated the extent to which the ERK cascade contributes to the proliferative response in bovine tracheal smooth muscle and showed that, although the PI3K pathway appeared to be essential for growth, activation of ERK was also required for a full mitogenic response [81]. Interestingly, EGF-induced PI3K activation in human airway smooth muscle cells occurs following association of PI3K with ErbB-2 [98], a member of the EGF receptor family that has no identified ligand. The mechanism of action is proposed to involve the EGF receptor causing transactivation of ErbB-2 and recruitment of PI3K, since no PI3K was found to associate with the activated EGF receptor itself [98].

In guinea-pig airway smooth muscle, sphingosine-1-phosphate, acting through an orphan Gi-coupled receptor termed EDG-1, was found to activate PI3K as measured in Grb2 immunoprecipitates. This study suggested that a Grb2/PI3K complex may act as an intermediate in ERK activation [97]. Another study in guinea-pig tracheal airway smooth muscle, using two structurally dissimilar PI3K inhibitors, demonstrated an essential role for this kinase in ERK activation. PI3K activity was increased in Grb2 immunoprecipitates from PDGF-stimulated cells, showing that PI3K may associate with Grb2 and offering a site at which PI3K may be integrated into the ERK cascade (fig. 4), [100]. It was suggested that this Grb2-associated PI3K could be a distinct isoform related to the activation of the ERK cascade.

As discussed above, it has previously been shown that, in airway smooth muscle, both cyclin D_1 expression [148] and DNA synthesis [80] involve ERK activation. Similar data, however, from the same investigators, using bovine tracheal smooth muscle, has shown that PDGF-stimulated cyclin D_1 transcription can occur independently of ERK activation, and is instead mediated by activation of the Rho family GTPase, Rac1 [149], believed to be activated as a consequence of PIP_3 generation. Thus both Rac1 and ERK appear to operate as distinct upstream regulators of PDGF-induced cyclin D_1 promoter activity, with Rac1 being neither required nor sufficient for ERK activation, as demonstrated by the failure of the MEK-1 inhibitor, PD98059, to prevent Rac1-induced responses. PDGF and Rac1-stimulated cyclin D_1 transcription was, however, prevented by treatment with reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitors and antioxidants, indicating the dependence on reactive oxygen species of the Rac1/NADPH oxidase pathway. Increasingly, as in many mesenchymal cells, it appears that cyclin D_1 expression is regulated by multiple effectors [150], and that ERK activation may initiate cyclin D_1 expression, whereas other signals such as PI3K, protein kinase B and Rac may provide continuing signals to the cyclin D_1 promoter. A similar sequence of events may be envisaged in airway smooth muscle, although the temporal aspects may be reversed. Hence, although pretreatment of airway smooth muscle with wortmannin prevented both EGF- and α-thrombin-stimulated DNA synthesis, in cases in which wortmannin was added 6 h after the cells were stimulated with mitogens, proliferation was no longer inhibited [151]. Thus in airway smooth muscle PI3K may be more important in modulating the early signals that lead to proliferation. By analogy, therefore, airway smooth muscle proliferation may be under the regulation of a number of signalling pathways with certain effectors being essential for initiating proliferation, whereas others act to modulate the ultimate magnitude and duration of the response. Much debate, however, remains before the identity of these critical and facilitatory effector pathways can be dissected with any certainty.

Effect of antiasthma drugs on cell cycle progression signalling pathways: identification of future target loci for therapeutic intervention

Glucocorticoids are the most effective therapy currently availably for the treatment of chronic asthma. Their main prophylactic action in reducing airway hyperresponsiveness in asthma is believed to be inhibition of recruitment of inflammatory cells and inhibition of release of pro-inflammatory mediators and cytokines from activated inflammatory and airway epithelial cells. The clinical efficacy of glucocorticoids in preventing or reversing structural changes in the airways, including smooth muscle hyperplasia/hypertrophy, is undefined (reviewed in [41]). In cell culture-based systems, however, glucocorticoids inhibit mitogen-stimulated proliferation of smooth muscle derived from bovine [152], rabbit [153] and human airways [154]. A recent study by Stewart and coworkers [155] has addressed the possible molecular mechanisms by which glucocorticoids could prevent induction of DNA synthesis by α-thrombin. Glucocorticoids were found to inhibit the passage of human airway smooth muscle cells through the restriction point (the stage in the cell cycle beyond which there is commitment of a cell to enter the S phase and to one round of DNA replication) by inducing a noncytotoxic, nonapoptotic arrest in the G_1 phase of the cell cycle [155]. This was associated with reduced levels of α-thrombin-stimulated phosphorylation of pRb by dexamethasone. Consistent with the induction of growth-arrest in G_1 and attenuated phosphorylation of pRb, glucocorticoids also reduced levels of α-thrombin-stimulated increases in cyclin D_1 expression. In the same study, dexamethasone was without effect on the activation of ERK by α-thrombin, suggesting that the major effect of glucocorticoids in preventing S phase traversal in these cells was parallel to, or downstream of, ERK activation [155]. However, the events that link activation of ERK to cyclin D_1 expression in airway smooth muscle are unknown, although activated glucocorticoid receptors are known to form a complex with AP-1, perhaps preventing its action on genes required for cell cycle progression to the S phase. In addition, glucocorticoids are powerful regulators of activation of nuclear factor-κB which has been implicated in the regulation of cyclin D_1 expression [156].

β_2-Adrenoceptor agonists represent another class of drugs used in the management of asthma which inhibit proliferation of mitogen-stimulated airway smooth muscle cells in culture [62, 157]. The action of β_2-adrenoceptor agonists is considered to be dependent largely on increases in intracellular cyclic adenosine monophosphate (AMP) levels, consistent with the antiproliferative effect of agents which directly increase cyclic AMP levels [158]. In another study, Stewart *et al.* [159] recently demonstrated that the β_2-adrenoceptor agonist, salbutamol, opposes the passage of human airway smooth muscle cells through the cell cycle restriction point. Consistent with this, salbutamol prevented induction of pRb hyperphosphorylation and cyclin D_1 protein expression by α-thrombin. However, no effect of salbutamol was found on cyclin D_1 mRNA, suggesting a post-transcriptional effect on cyclin D_1, perhaps involving accelerated degradation of the cyclin D_1 protein [159]. In contrast to the lack of effect of glucocorticoids on ERK activity [155], salbutamol prevented ERK activation between 5 min and 8 h following stimulation with α-thrombin, consistent with earlier reports that sustained ERK activation throughout G_1 is necessary for cell cycle traversal [74, 75, 78]. An additional mechanism that was not investigated in this study, but has been reported previously by these authors [160], was the salbutamol-induced expression of $p27^{kip1}$, a cdk protein inhibitor which prevents phosphorylation of pRb and cell cycle progression by inhibiting cdk activity in the mitogen-activated cyclin D_1/cdk4 complex.

Concluding remarks

Although asthma typically involves reversible airflow obstruction, in some asthmatics this obstruction is less reversible. In such patients, the obstruction is associated with persistent and largely irreversible structural changes in the airway wall, an important component of which is the increased mural smooth muscle content that arises due to repeated and intense stimulation of the smooth muscle by contractile agonists, pro-inflammatory mediators and polypeptide growth factors. Recently, it has been hypothesized

that the contribution that this increase in muscle mass makes to the pathogenesis of chronic asthma may not be limited to simple geometric obstruction (by increased airway wall thickening), but may also involve reversible phenotypic modulation of the muscle from a contractile to a more synthetic/proliferative state in which additional functions such as the production and release of pro-inflammatory mediators, growth factors and extracellular matrix elements are more apparent.

Although the precise events mediating these changes in airway smooth muscle and the role such changes may play in chronic asthma remain to be defined, the late 1990s have seen a great deal of new information, which has addressed the cellular mechanisms of proliferation and, most recently, altered phenotype in airway smooth muscle become available. It is now known that airway wall smooth muscle is not composed of a homogeneous population of cells, and that altered cellular heterogeneity may be an important factor in the pathogenesis and progression of asthma. It is also known from *in vitro* studies that, although many mediators can increase smooth muscle proliferation, similarities are present across species in the intracellular mechanisms activated by these mediators. The potential effects, however, of smooth muscle phenotypic modulation on intracellular signalling events are unknown. For the moment, it appears that diverse extracellular stimuli can induce cell growth by activation of common intracellular signalling pathways. This review has focused on three such pathways whose co-ordinated activation of proteins appears critical for transduction of the signal events necessary for the cellular proliferation of airway smooth muscle. The key players in the initiation and regulation of the proliferative response of airway smooth muscle appear to include extracellular-signal regulated kinase-, protein kinase C- and phosphoinositide 3-kinase-dependent pathways. Common to each of these pathways is the activation of Ras proteins (fig. 5), which may represent a point of convergence, and may thus be critical in the transduction and integration of the mitogenic signal from agonists that act through both receptor tyrosine kinase-linked and G protein-coupled receptors. The fate of the airway smooth muscle cell, therefore, appears to lie in its ability to co-ordinate the temporal aspect and magnitude of the signal inputs from these multiple effector pathways. Additional factors that may co-ordinate the mitogenic signal, such as the sequestration and subcellular localization of various signalling molecules and complexes, must also now be considered. The details of how all this is achieved will be the fruits of further investigation.

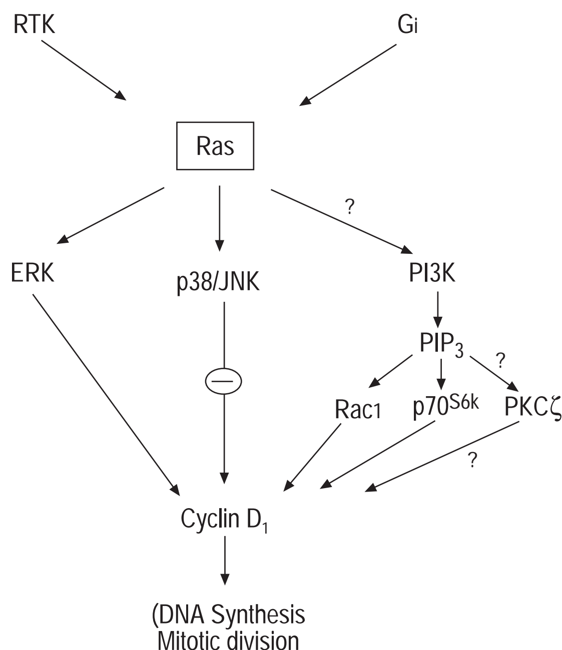

Fig. 5. – Involvement of Ras in the activation of multiple early signalling pathways for proliferation in airway smooth muscle. Mitogen-stimulated activation of receptor tyrosine kinases (RTK) and inhibitory G protein (Gi)-coupled receptors results in activation of Ras proteins in airway smooth muscle. The downstream effectors of Ras activation involve both positive and negative regulators of expression of cell cycle progression factors such as retinoblastoma protein (pRb) and cyclin D_1. Promitogenic Ras-dependent signals operate through both extracellular signal-regulated kinase (ERK)-dependent and ERK-independent mechanisms. Ras-dependent ERK-independent pathways in airway smooth muscle include activation of the phosphoinositide 3-kinase (PI3K), $p70^{S6k}$ and Rac1/ reduced nicotinamide adenine dinucleotide phosphate oxidase pathway, although direct activation of PI3K by Ras proteins has not been demonstrated in airway smooth muscle. Negative Ras-dependent signals involve activation of the p38 mitogen-activated protein kinase pathway [161]. Thus p21 Ras appears to be a unique and critical signalling molecule that integrates both RTK and Gi protein-coupled receptor-dependent pathways and their downstream effectors. Future therapeutic strategies for preventing inapprpriate proliferation of airway smooth muscle in asthma will undoubtedly focus on understanding the role of this molecule. JNK: Jun amino terminal kinases; PIP_3: phosphatidylinositol 3,4,5-triphosphate; PKC: protein kinase C; DNA: deoxyribonucleic acid.

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