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Interleukin 13 Increases Contractility of Murine Tracheal Smooth Muscle by a Phosphoinositide 3-kinase p110δ Dependent Mechanism

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d) **ABBREVIATIONS**: AHR, airway hyperresponsiveness; CCh, carbachol; DMSO, dimethylsulfoxide; IC87114, 2-(6-aminopurin-9-ylmethyl)-5-methyl-3-*o*-tolyl-3H-quinazolin-4-one; IL-13, interleukin 13; LY, LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one); OVA, ovalbumin; PI3K, phosphoinositide 3-kinase.

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ABSTRACT

The Th2 cytokine interleukin (IL-) 13 can elicit a number of responses consistent with a key role in the pathogenesis of asthma. We have utilised pharmacological and genetic approaches to demonstrate the role of signalling *via* the class I phosphoinositide 3-kinase p110δ isoform in IL-13-induced hyperresponsiveness of murine tracheal smooth muscle contractility in vitro. IL-13 treatment of tracheal tissue is associated with an early activation of phosphoinositide 3-kinase (PI3K), as assessed by phosphorylation of Akt. Tracheal smooth muscle contractility is enhanced by overnight incubation with IL-13, resulting in increased maximal contractions (Emax) to carbachol (CCh) and KCl. Inhibition of PI3K by the non-isoform selective inhibitors wortmannin or LY294002, or the selective inhibitor of the PI3K p110δ isoform IC87114, prevented IL-13-induced hyperresponsiveness. Consistent with a role for PI3K p110δ in IL-13-induced hyperresponsiveness, IL-13 was unable to induce hyperresponsiveness in tissues from mice expressing the catalytically inactive form of p110δ ($p110\delta^{D910A}$). These data indicate that IL-13 contributes towards tracheal smooth muscle hyperresponsiveness *via* the PI3K p110δ isoform. In addition to previously reported effects on airway inflammation, inhibition of PI3K p110δ may be a useful target for the treatment of asthma by preventing IL-13-induced airway smooth muscle hyperresponsiveness.

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Interleukin (IL-) 13 has been implicated as a key cytokine in the pathogenesis of allergy and asthma (Wills-Karp, 2004). Pulmonary expression of IL-13 induces an asthma-like phenotype in mice, including a mononuclear and eosinophilic inflammatory response, mucus cell metaplasia, airway fibrosis, eotaxin production, airways obstruction, and nonspecific airway hyperresponsiveness (AHR) (Zhu et al., 1999). In addition to enhancing polarisation of T-lymphocytes to a Th2 phenotype and promoting B-cell synthesis of IgE (Hajoui et al., 2004; Levy et al., 1997), IL-13 may contribute towards asthma *via* tissue remodelling (Kumar et al., 2002), epithelial activation (Lordan et al., 2002), decreasing β2 adrenoceptor function (Laporte et al., 2001) and enhancing contractility of airway smooth muscle (Tliba et al., 2003). Although several Th2 cytokines have been implicated in antigen-induced AHR, IL-13 appears to play a pre-eminent role. Targeted deletion of IL-13 prevents expression of AHR in allergen challenged mice, despite maintenance of elevated IL-4 and IL-5 release (Walter et al., 2001). Similarly, neutralisation of IL-13 using IL-13 receptor constructs or antibodies reduces AHR without influencing IL-5 levels (Grünig et al., 1998; Eum et al., 2005).

IL-13 binding to the IL-13 receptor results in activation of intracellular signal transduction cascades. While most signalling studies have concentrated on the Janus kinase/ signal tranducers and activators of transcription (STAT)-6 pathway, IL-13 also activates phosphoinositide 3-kinase (PI3K) and downstream effector molecules (Ceponis et al., 2000; Hershey, 2003; Wills-Karp, 2004; Wright et al., 1997). PI3K signalling and its putative roles in lung disease have been extensively reviewed (Ito et al., 2007; Medina-Tato et al., 2007; Vanhaesebroeck et al., 2001). The PI3K family is divided into three classes (I, II, and III) based on their different isoform structure and substrate specificity. PI3Ks phosphorylate the D-3 position of the inositol ring of target lipids. Class IA and IB PI3K are heterodimeric enzymes composed of a regulatory

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adapter (accessory) subunit coupled with a tightly bound catalytic subunit. Class IA catalytic subunits include p110α, β and δ while the class IB catalytic subunit is p110γ. Class I PI3K isoforms catalyze the phosphorylation of phosphatidylinositol-(4,5)-bisphosphate (PIP_2) to form phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) in response to activation of either receptor tyrosine kinase or G-protein-coupled receptors, which ultimately regulate cell growth, differentiation, survival, proliferation, migration, and cytokine production. The p110α and p110ß isoforms of the catalytic subunit are ubiquitously expressed and genetic knockout leads to early embryonic lethality, while animals lacking p110δ exhibit a high degree of normal development and growth. p110δ is expressed largely in circulating hematogenous cells and endothelial cells, but expression of p110γ is leukocyte restricted.

PI3K may contribute to the pathogenesis of asthma by effecting the recruitment, activation, and apoptosis of inflammatory cells (Medina-Tato et al., 2007). Administration of wortmannin or LY294002, two broad-spectrum inhibitors of PI3K, attenuates inflammation in murine models of allergic asthma (Ezeamuzie et al., 2001; Kwak et al., 2003). Intratracheal administration of LY294002 significantly inhibits most of the pathological characteristics of the mouse asthma model, including increased eosinophil counts and eotaxin, IL-5 and IL-13 levels in bronchoalveolar lavage fluid. Furthermore, lung tissue eosinophilia, airway mucus production and AHR to inhaled methacholine were all significantly suppressed (Duan et al., 2005). Although these studies with broad-spectrum inhibitors provide good evidence for a role for PI3K in allergic airway dysfunction, these inhibitors do not distinguish among the four class I PI3K isoforms (Davies et al., 2000). The development of isoform selective inhibitors, as well as genetically modified mice, allow the characterisation of the different roles of individual PI3K isoforms in airway disease.

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In smooth muscle, PI3K is implicated in the enhancement of agonist-induced contraction, as evidenced by the ability of pharmacological inhibitors or molecular manipulations of PI3K to reduce agonist-stimulated contraction of tissue from hypertensive rats (Northcott et al., 2005) or insulin-treated airway smooth muscle (Schaafsma et al., 2007). In addition to contributing toward the AHR seen in asthma, airway smooth muscle cells are potentially linked with many other features of asthma, including the production of cytokines and inflammatory mediators involved in tissue remodelling.

The present study examines the role of the PI3K signalling pathway in IL-13-induced hyperreactivity of murine tracheal smooth muscle. We provide evidence that a PI3Kδ-dependent mechanism plays a key role in IL-13-induced airway smooth muscle hyperresponsiveness, and modulation of this pathway may provide a useful therapeutic target in the treatment of respiratory disease.

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Materials and Methods

Animals. Breeding and maintenance of animals was according to UK Home Office regulations and guidelines for the care and welfare of laboratory animals, and fed with standard rodent chow and water *ad libitum.* Inhibitor experiments were carried out with male 8 -10 weeks old CD1 strain mice (University of Bath). In experiments using tissue from genetically modified animals, mice (male or female, 6-8 weeks) expressing a catalytically inactive p110δ isoform of PI3K ($p110\delta^{D910A}$) and matched DO11.10 controls (Okkenhaug et al., 2002) were provided by Dr Klaus Okkenhaug, Babraham Institute, Cambridge, UK.

Tissue preparation and tracheal organ culture. Animals were killed by exposure to a rising concentration of CO_2. The thorax and the ventral surface of the neck were opened by a mid-line longitudinal incision and the trachea from the larynx to the carina was rapidly removed. The oesophagus was carefully separated and the trachea was cleared of loose connective tissue, and divided into 2 segments. Tissues were placed individually in multiwell plates containing Dulbecco's modified Eagle's medium (containing D-glucose 25 mM, sodium pyruvate 1 mM, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2 M L-glutamine, 2.5 μg/ml fungizone and 0.1% w/v bovine serum albumin (Adner et al., 2002)). Tracheal segments were incubated at 37°C in a humidified CO_2 gassed incubator in the presence or absence of IL-13. The effect of PI3K inhibition was assessed by treating tracheal rings with wortmannin, LY294002 (both from Sigma-Aldrich, Poole, UK) or IC87114 (ICOS Corporation, Bothell, WA, USA) before or after IL-13 addition. These inhibitor concentrations were chosen based on their in vitro potencies (Ito et al., 2007; Wright et al., 1997) and verified using in vitro lipid kinase assay (below). Dimethyl sulfoxide- (DMSO, 0.05% v/v) treated tracheal rings served as vehicle controls in inhibitor experiments.

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Measurement of tracheal smooth muscle contractility. Trachea smooth muscle reactivity was assessed in temperature-controlled (37 °C) organ baths containing Krebs-Henseleit buffer solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11.1 (Moffatt et al., 2004), continuously bubbled with 5% CO_2 and 95% O_2. Tracheal segments were mounted in organ baths between two metal hooks and connected to a K30 force displacement transducer (Hugo Sachs Elektronik, March, Germany) under approximately 5 mN resting tension. Isometric contractile responses were recorded with a MacLab/4e and Quad bridge amplifier linked to a PC running Chart 4 software for Windows (ADIstruments Ltd, Oxon, UK).

Immunoblot analysis of phospho-Akt. Tracheal tissue was homogenized in lysis buffer (Tris–HCl 50 mM pH 7.5, NaCl 150 mM, EDTA 5 mM, Nonidet P40 1% v/v, Na_3VO_4 1 mM, Na_2MoO_4 1 mM, NaF 10 mM, leupeptin 10 μg/ml, aprotinin 10 μg/ml, soyabean trypsin inhibitor 10 μg/ml, phenylmethylsulphonyl fluoride 1 mM and pepstatin A 1 μg/ml) with 10% v/v glycerol. Tracheal lysates were centrifuged at 15,400 g for 15 min at 4 °C, and the supernatant was collected. Protein concentration was quantified using the Bradford assay (BioRad, Hercules, CA) and equal amounts of protein (30 μg per lane) were subjected to electrophoresis on SDS-polyacrylamide gels (12% w/v). Proteins were transferred to nitrocellulose membranes (Whatman, UK) by semidry transfer blot (Transblot SD cell, Bio-Rad, CA, USA), and blots were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS)-Tween (Tris HCl 20 mM pH 7.5, NaCl 150 mM, pH 7.4 and 0.1% v/v Tween20) for 1 h. Membranes were then incubated with primary antibody (rabbit phospho-Akt (Ser^{473}) antibody, 1:700 dilution; Cell Signalling Technologies, MA, USA) overnight at 4 °C. The blots were then washed three times in TBS-Tween before incubated with a horseradish peroxidase-conjugated anti-rabbit IgG

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secondary antibody (1:7000 dilution, Dako, Glostrup, Denmark) at 20 °C for 2 h. Bound antibody complexes were detected using chemiluminescence reagent (Amersham Biosciences, Buchinghamshire, UK) according to the manufacturer's instructions. Membranes were then reprobed with a pan-Akt (C-20) goat polyclonal antibody (1:1000 dilution, Santa Cruz, CA, U.S.A.) and anti-goat secondary antibody for pan-Akt (1:10000 dilution, Dako, Glostrup, Denmark) to confirm equal loading.

In vitro lipid kinase assay. As tracheal tissue did not yield sufficient material to assess the activity of individual PI3K isoforms, whole murine lung tissue was utilised to determine the specificity of the inhibitors used. After euthanasia, lungs and heart were thoroughly washed with ice-cold phosphate-buffered solution (PBS) through the pulmonary artery to reduce intravascular erythrocyte and leukocyte content. The lungs were carefully dissected and washed with PBS before being chopped into fragments of less than 4 mm^3. Approximately 100 mg of lung fragments were homogenised in lysis buffer, centrifuged at 13,000 g at 4 °C for 30 min to eliminate debris and the supernatants were then collected in clean plastic tubes. The lipid kinase activity of PI3K was measured by an in vitro lipid kinase assay which detects the transfer of radiolabeled γ-phosphate of ATP to the D-3 position of phosphoinositides (PI), resulting in the formation of (γ^{32}P)-labelled PI(3)P (Ward et al., 1992; Wright et al., 1997). Briefly, PI3K isoforms were immunoprecipitated and captured by the addition of rabbit polyclonal antibody (Santa Cruz, CA, U.S.A) against PI3K p110α, p110β, p110δ or p110γ. Protein G beads were then added and the samples were rotated for 1 h at 4 °C. The beads were captured by centrifugation at 13,000 g for 1 min at 4 °C and samples were then washed and pre-treated with inhibitors prior to assessment of lipid kinase activity by addition of ATP reaction buffer (0.88 mM ATP, 20 mM $MgCl_2$, and 5-10 μCi [γ^{32}P]-ATP. Reactions were terminated after 10 minutes by the addition of 5 M HCl and

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lipids extracted with chloroform:methanol (1:1 v/v). The lower chloroform layer was removed and spotted onto 1% potassium oxalate-treated thin layer chromatography (TLC) plates. Lipids were resolved by TLC in chloroform:methanol:water:ammonium hydroxide (60:47:11.3:2 v/v/v/v) and visualised by autoradiography at -80°C before plates were stained with iodine to confirm equal loading.

Statistical Analysis. Contractions evoked by KCl and CCh are expressed as mN/mg tissue wet weight and all values are presented as mean ± standard error of mean (s.e.m.). Non-linear regression analysis using GraphPad Prism (v4; GraphPad Software, San Diego, CA, USA) was used to determine Emax. Comparisons among groups were performed by Student's paired t-test or ANOVA with Dunnett's or Tukey-Newman-Keuls post-hoc tests as appropriate.

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Results

Enhancement of KCl- and CCh -induced contraction of murine tracheal smooth muscle by IL-13. Incubation of murine tracheal rings with IL-13 for 24 h potentiated KCl (10-100 mM) and CCh (10^{-8}-10^{-5} M)-induced contractions (Fig. 1A and B). The agonist Emax of the concentration-response curve was significantly higher in rings pre-treated with IL-13 (10 ng/ml or 100 ng/ml) compared with controls, with Emax values for KCl and CCh increased approximately 1.6-2 fold (Table 1). Although IL-13 elevated the maximal response, limited changes in EC50 values were observed (Table 1), indicating that that IL-13 appeared to increase smooth muscle contractility rather than inducing increased sensitivity to low concentrations of contractile agents.

IL-13-induced Akt phosphorylation in tracheal lysate. The ability of IL-13 to activate PI3K signalling in murine tracheal tissue was assessed by assessing the phosphorylation Akt, a downstream target of PI3K. IL-13 (100 ng/ml) induced a rapid phosphorylation of Akt Ser^{473} in murine tracheal tissue, with increased phospho-Akt detected after 2-10 min exposure (Fig. 2). Akt phosphorylation was also observed after longer exposure to IL-13 (data not shown).

Sensitivity of PI3K isoforms to LY294002 and IC87114 in murine lung. The sensitivity of the PI3K Class I isoforms expressed in lung cells to PI3K inhibitors was assessed by lipid kinase assay. The different class I isoforms from whole lung cell lysates were immunoprecipitated and their ability to transfer radiolabeled γ-phosphate in the presence of inhibitors assessed. The lung lysates contained all four class I PI3K isoforms, as determined by immunoprecipitation. As shown in Fig. 3, LY294002 inhibited all four isoforms with similar potency. However, IC87114 demonstrated selectivity for p110δ activity, with p110α, p110β and p110 γ isoforms showing limited inhibition even at the highest concentration (30 μM) of this compound examined. It is assumed that the p110γ isoform detected in these whole

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lung lysates was derived from intravascular and tissue resident leukocytes, which are not likely to be present in significant numbers in the tracheal tissue used in other parts of this study.

PI3K is involved in IL-13-induced hyperresponsiveness. To examine the potential role of PI3K in IL-13-induced hyperresponsiveness, the effects of two structurally distinct non-isoform selective PI3K inhibitors, wortmannin (100 nM) and LY29402 (10 μM), were assessed. Addition of these inhibitors 30 min prior to IL-13 addition prevented the induction of hyperresponsiveness, reducing Emax values for both KCl and CCh to that of drug vehicle-treated control tissues (Fig. 4, Table 2). Under the conditions used, neither wortmannin nor LY29402 had any effect on the contractility of murine tracheal smooth muscle in tissues not pretreated with IL-13 (Table 2). Furthermore, in experiments where the irreversible PI3K inhibitor wortmannin (100 nM) was added after 24 h incubation with IL-13, significant hyperresponsiveness was still observed, resulting in Emax values for KCl and CCh of 2.8 ± 0.3 ($p<0.05$) and 4.1 ± 0.4 ($p<0.001$, n=6) compared with control values of 1.7. ± 0.1 and 2.2 ± 0.1 (n=7) respectively.

Role of PI3Kδ in IL-13-induced hyperresponsiveness. To determine if the PI3K p110δ isoform contributes to IL-13-induced hyperresponsiveness in murine tracheal rings, tissues were treated with the selective PI3Kδ inhibitor IC87114 30 min before IL-13 addition (Fig. 5). IC87114 (10 μM) was able to completely inhibit the ability of IL-13 to elicit hyperresponsiveness to either KCl or CCh. In control experiments, this compound did not reduce tracheal smooth muscle contractility in the absence of IL-13 treatment, giving Emax values for KCl and CCh of 1.7 ± 0.3 and 2.6 ± 0.5 respectively, compared with 1.7 ± 0.2 and 2.2 ± 0.1 in vehicle control treated tissues.

Effects of IL-13 in tracheal rings from p110δ^{D910A} mice

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To investigate further the role of PI3Kδ in IL-13-induced hyperresponsiveness, tracheal rings were obtained from mice expressing p110δ^{D910A}, a catalytically inactive form of p110δ (Okkenhaug et al., 2002). These mice were generated by point mutation instead of deletion to prevent changes in the expression levels of the other PI3K catalytic and regulatory subunits. IL-13 was unable to evoke hyperresponsiveness in rings isolated from p110δ^{D910A} mice compared with control rings isolated from same mice (Fig. 6, Table 3). However, IL-13 did elicit hyperresponsiveness in response to KCl and CCh in tracheal rings isolated from control DO11-10 mice.

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Discussion

IL-13 plays a central role in the development of airway inflammation and bronchial hyperresponsiveness in asthma (Wills-Karp, 2004). A number of reports have shown that IL-13 may exert its deleterious effects in asthma by acting directly on resident airway cells, including epithelial cells and airway smooth muscle cells (Kuperman et al., 2002; Syed et al., 2005; Tliba et al., 2003). Our study supports and extends these findings. We demonstrate the role of resident cells in IL-13-induced increases in airway smooth muscle contractility in response to CCh and KCl, which act *via* G-protein coupled receptor-dependent and voltage gated ion channel-dependent mechanisms respectively. Since contractile responses to these agents were influenced to a similar extent by IL-13, it appears likely that the modulation is at the level of the smooth muscle contractile apparatus, rather than influencing receptor density or transduction. Fredberg (2004) defined the hyperreactivity component of airway hyperresponsiveness, represented by Emax, as the ability of the airways to narrow excessively and added that it accounts for the morbidity and mortality associated with asthma. Our study showed that the effects of IL-13 on contractility were principally to increase Emax, and no significant changes in EC50 were observed.

The mechanism by which IL-13 induces airway smooth muscle hyperresponsiveness is incompletely characterised. It is possible that IL-13 may be acting by the induction of other cytokines. IL-13-induced hyperresponsiveness can be inhibited by anti-IL-5 antibody in the rabbit trachea, and IL-13 upregulates IL-5 expression in human tissue (Grunstein et al., 2002). Since IL-5 has been shown to enhance responsiveness of human bronchus (Rizzo et al., 2002), it is possible that in our study PI3K inhibitors are acting to suppress IL-13-induced IL-5 release. However, overexpression of IL-13 in the mouse lung does not result in IL-5 (or IL-4) upregulation (Zhu et al., 1999), IL-13-induced in vivo AHR is maintained in IL-5 knock-out mice (Yang et al., 2001), and antigen-induced AHR is inhibited by anti-IL-13 but not anti-IL-5

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treatment (Grünig et al., 1998). At the level of signalling, IL-13 enhances agonist-stimulated calcium signalling in human airway smooth muscle cultures (Tliba et al., 2003), an effect that may be *via* an upregulation of cyclic adenosine diphosphate ribose (Deshpande et al., 2004). Several lines of evidence in our study implicate PI3K signalling in IL-13-induced hyperresponsiveness. IL-13 treated tracheal segments express phosphorylated Akt. However, our immunoblot analysis showed IL-13 induced a very early (after 2 minutes, as assessed by PI3K-dependent phosphorylation of Akt) activation of PI3K, while increased responsiveness was not observed until overnight incubation (Tliba et al., 2003; and own unpublished studies). In an *in vivo* allergen challenge model, Lee et al. (2006) noted Akt phosphorylation at 1, 24, 48, and 72 h after ovalbumin (OVA) inhalation, likely as a consequence of an ongoing inflammatory response and persistent local cytokine production. Duan et al. (2005) reported that intratracheal administration of LY294002 significantly inhibited OVA-induced increases in total cell counts, eosinophil counts, and IL-5, IL-13, and eotaxin levels in bronchoalveolar lavage fluid, and dramatically inhibited OVA-induced tissue eosinophilia and airway mucus production. This was associated with a significant suppression of OVA-induced AHR to inhaled methacholine. Importantly, Duan's study confirmed that LY294002 markedly attenuated OVA-induced serine phosphorylation of Akt, a downstream target of PI3K. These findings support studies showing attenuated eosinophilic airway inflammation and airway hyperresponsiveness by LY294002 and wortmannin in murine asthma models (Ezeamuzie et al., 2001; Kwak et al., 2003).

Our study provides functional evidence for the role of PI3K in the regulation of IL-13-induced hyperresponsiveness to KCl and CCh, established by pharmacological blockade of PI3K activity using wortmannin and LY294002. IL-13-induced hyperresponsiveness in isolated murine tracheal rings was prevented by these non-isoform selective inhibitors. Recent *in vivo* studies support a role for PI3K p110δ for IL-13-induced AHR. Lee et al. (2006) demonstrated

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that p110δ was the main component of class I PI3K-dependent, allergen-induced Akt activation and inflammation in the lung and IC87114 significantly suppressed OVA-induced AHR to methacholine *in vivo*. Nashed et al. (2007) also established that hyperresponsiveness to inhaled methacholine was markedly attenuated in p110δ-inactivated mice after allergen challenge, but in their model the major defect arising from p110δ deficiency was believed to be a qualitatively altered immune capacity, rather than alterations in structural cells. Our findings, using isolated tracheal rings treated with the p110δ-selective inhibitor, as well as using tissue from animals expressing the catalytically inactive PI3K subunit $p110\delta^{D910A}$, indicate a crucial role of p110δ in inflammation-independent induction of AHR by IL-13. Hence, while previous studies implicate the involvement of the PI3K p110δ isoform in the inflammatory component of AHR, our findings indicate an important role in IL-13-induced airway smooth muscle hyperresponsiveness independent of effects mediated by infiltrating immune cells. These dual actions of IL-13-stimulated p110δ activity, both in airway inflammation, as well as in the direct effects on structural cells shown in the present study, point to p110δ as an attractive target for the treatment of airway disease. An important feature of our study is that PI3K was involved only in IL-13-enhanced contractility, since contractility of control tissues treated with inhibitors, or tissue expressing $p110\delta^{D910A}$, had similar responsiveness to normal tissues, whereas IL-13-induced enhanced contractility responses were reduced to those of tissues not exposed to IL-13. Furthermore, treatment of tissues with wortmannin after 24 h exposure to IL-13 was unable to reduce the enhanced responsiveness, suggesting a role for PI3K in the development of hypercontractility, rather than in the contraction response *per se*.

PI3K may regulate smooth muscle contractility by several mechanisms, although most studies have been carried out in vascular smooth muscle. All four class I PI3K isoforms are potentially able to stimulate L-type calcium channels in portal vein myocytes (Macrez et al., 2001).

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Inhibition of PI3K with LY294002 decreases agonist- and KCL-evoked contractile responses in porcine carotid artery by calcium dependent and independent pathways (Su et al., 2004). p110δ is implicated in enhanced contractility of angiotensin II-treated aortas from diabetic rats (Koyabashi et al., 2006) or aorta from spontaneously hypertensive rats (Northcott et al., 2005). Insulin, a well-characterised activator of PI3K, increases bovine tracheal smooth muscle contractility and expression of contractile proteins *via* an LY294002-sensitive mechanism (Schaafsma et al., 2007), but prolonged (>2 days) exposure to insulin was required before enhanced responsiveness was observed. Nevertheless, it is possible that significant transcriptional events occur in the time-course of our experiments.

In summary, our results provide clear evidence that PI3Kδ signalling is required for IL-13-induced tracheal smooth muscle hyperresponsiveness. This effect may be attributed to the direct effect of IL-13 on resident airway cells and signalling *via* this pathway may be a pharmacological target for the treatment of the airway hyperresponsiveness seen in asthma.

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Footnotes

a) This work was supported by an Egyptian Government Scholarship (MM/1704) to H.S.M.F. and a Mexican Government Scholarship (# 187285) to D.A.M.T. . Some of this work has been presented in poster format at the 5th James Black Conference in Crieff, Scotland, October 2007.

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Legends for Figures

Fig. 1. IL-13 enhances KCl- and CCh-induced contraction of tracheal rings. Cumulative concentration–response curves to (A) KCl (n=12) and (B) CCh (n=12) following incubation in the absence or presence of IL-13 (10 or 100 ng/ml, 24 h). Data are expressed as mean ± s.e.m. mN/mg tissue wet weight. One-way ANOVA followed by Dunnett's test was performed to determine the statistical significance of differences between Emax values of control and IL-13-treated tissues. **, $p < 0.01$; compared with non-IL-13 treated tissue.

Fig. 2. Effect of IL-13 on Akt phosphorylation in tracheal rings. Tracheal rings from 8 CD1 mice treated with IL-13 (100 ng/ml) for 2 -10 min were homogenized in ice-cold lysis buffer. Proteins (30 μg per lane) were separated by SDS-PAGE and probed with anti-phospho-Akt and anti-Akt antibodies before detection by enhanced chemiluminescence. (A) Immunoblot from one experiment representative of three. After probing for phospho-Akt, blots were reprobed for Akt to determine equal loading. (B) Densitometric analysis of phosphor-Akt expression. Results are expressed as % phospho-Akt as compared with total Akt, as determined using Labimage software (Lapelan Bio-imaging Solution, Halle, Germany). Bars indicate the mean ± s.e.m. density ratio from three independent experiments. ** Significant difference from control, $p < 0.01$.

Fig. 3. Inhibition of class I PI3K catalytic isoforms by LY294002 and IC87114 in murine lung tissue lysates. Immunoprecipitates (IP) for p110α, p110β, p110δ and p110γ were obtained from pooled murine lung lysates from 8 animals and assayed for in vitro lipid kinase activity after a 30 min pretreatment with vehicle (V) or increasing

concentrations (1-30 μM) of LY294002 or IC87114. Reactions products were extracted, fractionated by TLC and examined by autoradiography. Results are representative of three independent experiments.

Fig. 4. Effect of PI3K inhibitors on IL-13-induced hyperesponsiveness to (A, B) KCl and (C, D) CCh. Tissues were incubated with drug vehicle (Control), wortmannin (100 nM) or LY294002 (10 μM) for 30 min prior to exposure to murine IL-13 (100 ng/ml) for 24 h before assessing tissue responses to contractile agents. Data from groups were expressed as mean ± s.e.m. of mN/mg tissue wet weight. One-way ANOVA followed by Dunnett's test was performed to determine the statistical significance of differences between Emax values. **, $p < 0.01$ compared with Control; ††, $p<0.01$ compared with IL-13 treated tissue.

Fig. 5. IC87114 prevents IL-13-induced hyperresponsiveness to (A) KCl and (B) CCh. Isolated murine tracheal segments were treated for 30 min with vehicle (Control) or with IC87114 (10 μM) then incubated for a further 24 h in the absence or presence of IL-13 (100 ng/ml). Data points indicate mean ± s.e.m for n=5-7 animals. One-way ANOVA followed by Dunnett's test was performed to determine the statistical significance of differences between Emax values; **, significantly different compared with Control, $p < 0.01$; ††, significantly different compared with tissues treated with IL-13 alone, $p<0.01$.

Fig. 6. Effect of IL-13 on responsivess of tissues from $p110\delta^{D910A}$ mice. Isolated murine tracheal segments from DO11-10 control or $p110\delta^{D910A}$ kinase-dead mice were incubated with IL-13 (100 ng/ml, 24 h) before assessment of responsiveness to KCl (A)

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or CCh (B). Data indicate mean ± s.e.m for n=6 mice. **, $p < 0.01$ compared with matched tissue not treated with IL-13, Student's paired t-test**.**

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Table 1. Effect of IL-13 on KCl and CCh - induced contractions in isolated murine tracheal rings.

Tissues isolated from CD1 mice and were incubated with media alone (controls, n=24) or IL-13 (10 and 100 ng/ml, n=12 per group) for 24 h prior to assessment of contractile responses to KCl or CCh. Values are mean ± s.e.m. of Emax values (mN/mg tissue wet weight) or pEC50 (-log[M]).

	KCl		**CCh**	
	Emax	**pEC50**	**Emax**	**pEC50**
Control	1.2 ± 0.1	1.43 ± 0.03	2.0 ± 0.2	6.53 ± 0.09
IL-13 (10 ng/ml)	2.2 ± 0.3 [a]	1.44 ± 0.03	3.8 ± 0.5[a]	6.54 ± 0.10
IL-13 (100 ng/ml)	2.7 ± 0.4 [a]	1.44 ± 0.03	3.8 ± 0.5 [a]	6.53 ± 0.09

[a] $p < 0.01$ compared with matched Control segments.

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Table 2. Effect of broad-spectrum PI3K inhibitors on IL-13-induced hyperresponsiveness in isolated tracheal rings.

Tissues were preincubated for 30 min with drug vehicle (Control, DMSO, 0.05% v/v), wortmannin (100 nM) or LY-249002 (10 μM). Media or IL-13 (100 ng/ml) was then added for a further 24 h before assessing contractility responses to KCl and CCh. Data are expressed as mean Emax (mN/mg wet weight tissue) ± s.e.m for n=5-7 rings from different animals.

	KCl	**CCh**
Control	1.7 ± 0.1	2.2 ± 0.1
Wortmannin	1.4 ± 0.2	1.8 ± 0.2
LY294002	1.9 ± 0.4	2.5 ± 0.4
IL-13	2.8 ± 0.3[a]	3.1 ± 0.2[b]
IL-13 + Wortmannin	1.3 ± 0.2[d]	1.8 ± 0.3[d]
IL-13 + LY294002	1.4 ± 0.1[d]	2.2 ± 0.3[c]

[a]$p < 0.05$; [b]$p < 0.01$; compared with Control

[c]$p < 0.05$; [d]$p < 0.01$; compared with tissues treated with IL-13 alone.

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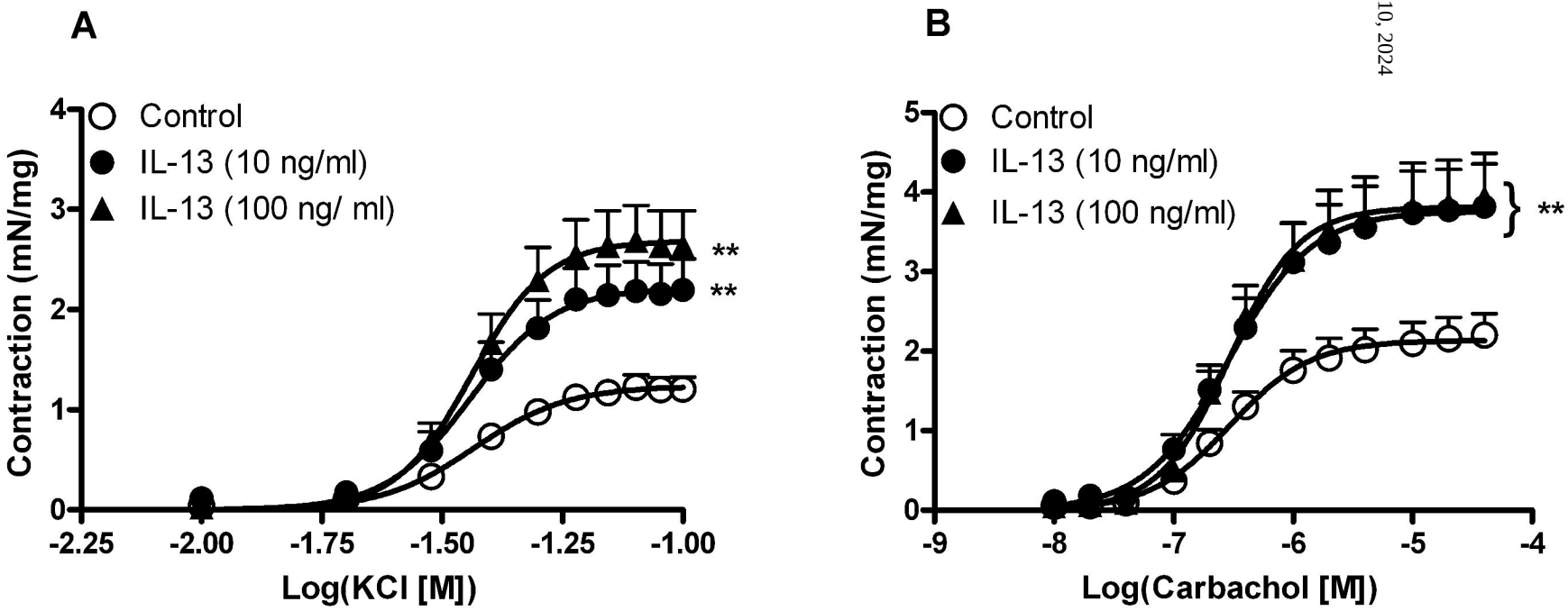
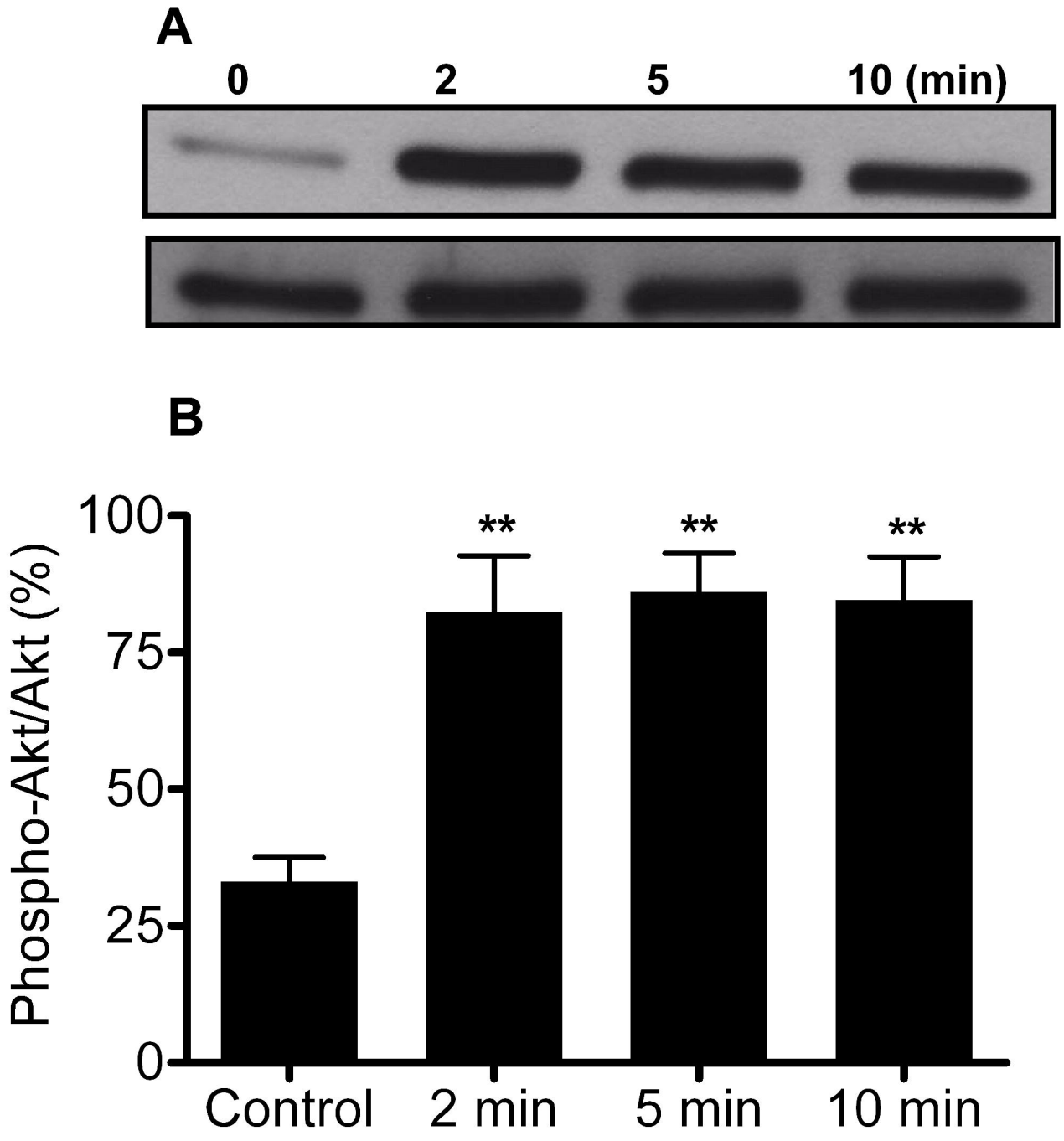
Table 3. Effect of IL-13 on KCl- and CCh-induced contractions in isolated murine tracheal rings of p110δ^{D910A} and DO11-10 mice.

Data are expressed as mean Emax (mN/mg tissue wet weight) ± s.e.m. for n=6 pairs of tracheal rings.

	KCl		CCh	
	Control	**IL-13**	**Control**	**IL-13**
DO11-10 control mice	1.8 ± 0.2	2.9 ± 0.2[a]	2.5 ± 0.3	3.7 ± 0.3[a]
p110δ^{D910A} mice	1.9 ± 0.3	1.8 ± 0.4	2.8 ± 0.3	2.4 ± 0.2

[a] $p < 0.01$ compared with non-IL-13 treated controls.





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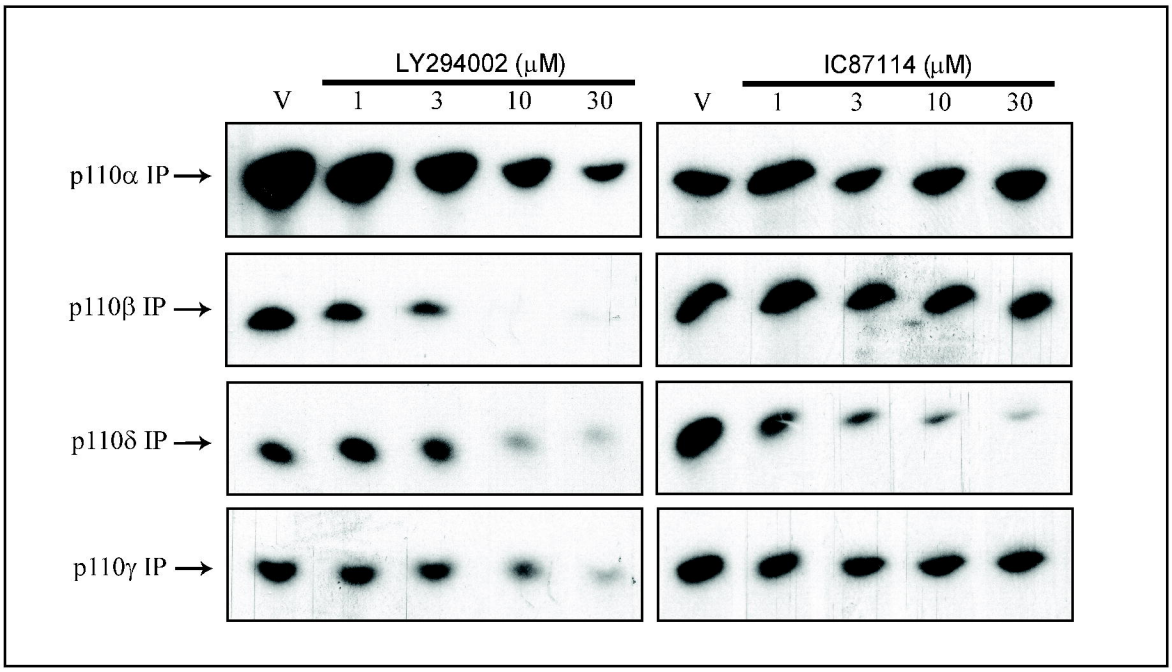

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