British Journal of Pharmacology (2003) 140, 1159–1162
www.nature.com/bjp

SPECIAL REPORT

IL-13 enhances agonist-evoked calcium signals and contractile responses in airway smooth muscle

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Growing evidence suggests that interleukin (IL)-13, a Th2-type cytokine, plays a critical role in the development of bronchial hyper-responsiveness (BHR), an essential feature of asthma, although the underlying mechanisms remain unknown. In the present study, we investigated whether IL-13 directly affects airway smooth muscle (ASM) function. In murine tracheal rings, IL-13 (100 ng ml^{-1}, 24 h) significantly increased both the carbachol- and KCl-induced maximal force generation without affecting ASM sensitivity. In cultured human ASM cells, IL-13 (50 ng ml^{-1}, 24 h) also augmented cytosolic calcium levels to bradykinin, histamine and carbachol by 60, 35 and 26%, respectively. The present study demonstrates that IL-13 may promote BHR by directly modulating ASM contractility, an effect that may be due to enhanced G protein-coupled receptor (GPCR)-associated calcium signaling.
British Journal of Pharmacology (2003) **140,** 1159–1162. doi:10.1038/sj.bjp.0705558

Keywords: Asthma; isometric tension; airway smooth muscle; Th2 cytokine; calcium metabolism

Abbreviations: IL, interleukin; BHR, bronchial hyper-responsiveness; ASM, airway smooth muscle; K–H, Krebs–Henseleit; GPCR, G protein-coupled receptor

Introduction Studies now suggest that cytokine-induced modulation of airway smooth muscle (ASM), an important effector tissue regulating bronchomotor tone, may play an important role in the development of bronchial hyper-responsiveness (BHR) in chronic lung diseases such as asthma and chronic obstructive pulmonary disease (reviewed in Amrani & Panettieri, 2002). The mechanisms by which cytokines promote BHR have not been clearly established; however, reports using cultured ASM cells showed that TNFα or interleukin (IL)-1β, proinflammatory cytokines found in the bronchoalveolar lavage of subjects with asthma, directly regulates agonist-associated calcium signaling, a critical element regulating ASM contraction (Amrani *et al*., 1995; Deshpande *et al*., 2003; Hunter *et al*., 2003). In addition, evidence using animal models of asthma shows that the Th2-type cytokine IL-13 may also play a critical role in promoting BHR, although the exact mechanisms are unknown (Grunig *et al*., 1998; Wills-Karp *et al*., 1998; Walter *et al*., 2001; Akbari *et al*., 2003). Recent studies, however, showed that IL-13 may exert its deleterious effects in asthma by directly altering gene expression in airway resident cells such as epithelial or ASM cells (Laporte *et al*., 2001; Lee *et al*., 2001; Kuperman *et al*., 2002; Venkayya *et al*., 2002).

In this study, we report that IL-13 directly alters ASM responsiveness by enhancing contractile agonist-induced contractility and calcium signals. Further examination of the mechanisms by which IL-13 regulates ASM function may offer new thera-peutic approaches to treat BHR associated with asthma.

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Advance online publication: 3 November 2003

Methods *Measurement of isometric force generation* Measurements of force generation using murine cultured tracheal rings were performed as described previously (Chen *et al*., 2003). Tracheae were supported longitudinally by a plexiglas rod with a stainless-steel pin into the base of a double-jacketed, glass organ bath filled with 10 ml of Krebs–Henseleit (K–H) solution at 37°C. The K–H solution contained (mM): 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 11.1 dextrose, 1.2 $MgSO_4$, 2.8 $CaCl_2$ and 25 $NaHCO_3$, and was continuously aerated with a 5% CO_2 and 95% O_2 mixture; a pH of 7.40–7.45 was established for the duration of the experiments. The upper support was attached by a loop of silk thread to an FT03 isometric transducer (Astro-Med, Inc., West Warwirck, RI, U.S.A.) and changes in tension of the rings were measured. Concentration–response curves were synchronously recorded with an MP 100WS system (BIOPAC Systems, Inc., Santa Barbara, CA, U.S.A.) and displayed on a Macintosh computer. All initial tensions of tracheal rings were set at approximately 0.5 g and stimulated with agonists after attainment of steady-state tension.

RT–PCR analysis RT–PCR analysis was performed as described previously (Chen *et al*., 2003). Briefly, total RNA was extracted from total murine tracheal rings by using the SV total RNA isolation system (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions. RT–PCR reactions were performed with the use of IL-13Rα1, IL-13Rα2 and β-actin primers for semiquantitative analysis as

described previously (Zheng *et al*., 2003). The PCR products (IL-13Rα1, 549 bp; IL-13Rα2, 217 bp; and β-actin, 241 bp) were resolved on 1.8% agarose-gel electrophoresis, stained with ethidium bromide and photographed.

Measurement of intracellular calcium concentration Calcium measurements were determined as described previously (Deshpande *et al*., 2003). Briefly, Fura-2-loaded human ASM cells grown on coverslips were mounted onto an open slide chamber, placed onto an inverted microscope and excited at 340 and 380 nm wavelength and emissions were collected at 510 nm wavelength using a CCD camera. The ratio of fluorescence intensities at 340 and 380 nm wavelength was determined and converted to the calcium concentrations using a standard curve. The net calcium responses to contractile agonists were calculated by subtracting the basal from that of the peak intracellular calcium concentration.

Data analysis Tension was calculated as milligram tensions per milligram trachea weight ($mg\,mg^{-1}$) and expressed as an individual percentage (%) of 10^{-5} M carbachol- or 100 mM KCl-evoked force of the cultured tracheal rings in the absence of IL-13 for contraction studies. The concentrations of agonists required to produce half-maximal contraction (pD_2) were determined with log values of the EC_{50}'s. All values were expressed as means ± s.e.m. Comparisons among groups with or without IL-13 were performed by a one-way analysis of variance followed by Student's unpaired *t*-test when appropriate. A *P*-value of less than 0.05 was considered significant.

Materials and reagents Recombinant murine and human IL-13 were purchased from R&D Systems (Indianapolis, IN, U.S.A.). Carbachol, bradykinin, histamine and Fura2/AM were purchased from Sigma (St Louis, MO, U.S.A.).

Results *IL-13 potentiates carbachol- and KCl-evoked force generation* Cultured tracheal rings generate force in response to carbachol and KCl in a concentration-dependent manner with pD_2 values of 6.62 ± 0.05 ($n = 18$) and 1.39 ± 0.07 ($n = 7$), respectively. In tracheal rings pretreated with $100\,ng\,ml^{-1}$ of murine IL-13, there was a significant increase in force generation induced by both carbachol and KCl as compared to those obtained from rings treated with diluent alone (Figure 1a and b). In IL-13-treated rings, the maximal tensions to carbachol and KCl were increased by 58 and 51% ($P < 0.05$), respectively. Neutralizing anti-IL-13 antibody ($5\,\mu g\,ml^{-1}$) completely prevented the IL-13-enhancing effect on agonist-evoked contraction (data not shown). In addition, the enhancing effect of IL-13 on the maximal force generation was not associated with changes in receptor affinity with pD_2 values of 6.69 ± 0.04 (carbachol, $n = 12$) and 1.42 ± 0.05 (KCl, $n = 6$). RT–PCR analysis revealed that both IL-13 receptors (IL-13Rα1 and IL-13Rα2) are expressed in murine tracheal rings (Figure 1c). These data show that IL-13 enhances maximal force generation induced by carbachol and KCl without altering receptor affinity.

IL-13 enhances agonist-evoked calcium signals Since cytokines have the capacity to enhance calcium signaling to a variety of contractile agonists in ASM cells (reviewed in Amrani & Panettieri, 2002), we examined whether human IL-13 also modulates agonist-induced calcium signals in Fura-2-loaded human ASM cells. As shown in Figure 2, IL-13 (24 h, $50\,ng\,ml^{-1}$) significantly increased calcium signals induced by

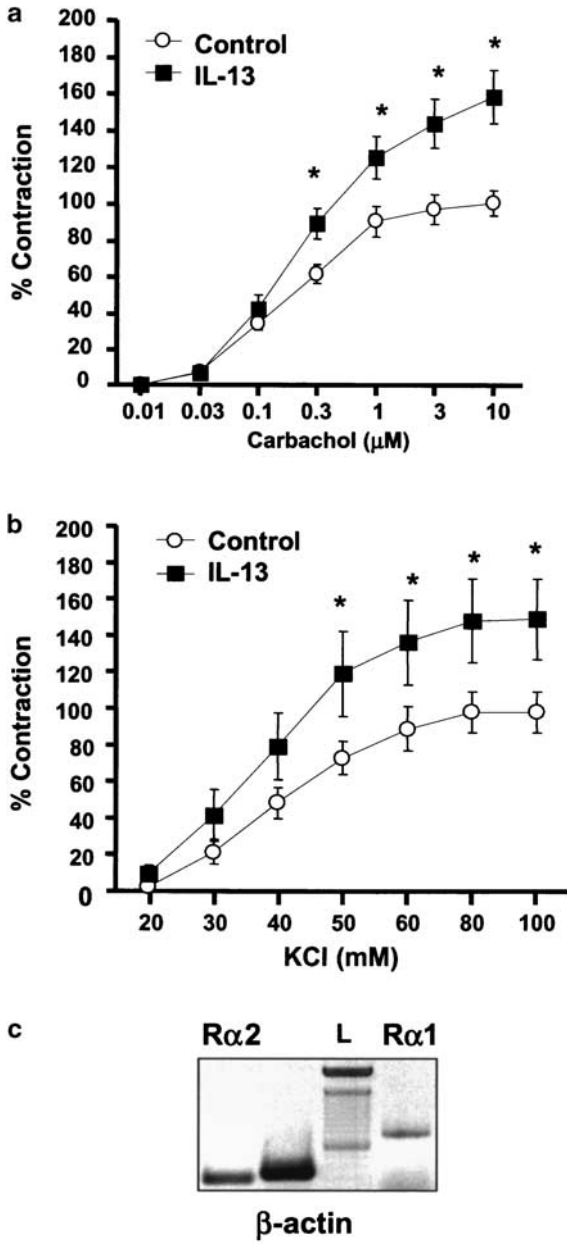

Figure 1 IL-13 enhances carbachol- and KCl-induced contraction in cultured trachea. (a) Cumulative concentration–response curves to carbachol were completed in cultured control ($n = 18$) or in the presence of IL-13 $100\,ng\,ml^{-1}$ ($n = 12$). (b) Cumulative concentration–response curves to KCl were completed in the absence ($n = 7$) and presence of IL-13 $100\,ng\,ml^{-1}$ ($n = 6$). All tension measurements from groups were expressed as mean ± s.e.m., $^*P < 0.05$ compared with rings treated with diluent alone. (c) Expression of IL-13 receptors in murine tracheal rings. Total RNA (1 μg) was subjected to RT–PCR with the primers for β-actin, IL-13Rα1 and IL-13Rα2 as described in Methods. PCR products were separated on 1.8% agarose gel and stained with ethidium bromide. L, 100 bp DNA ladder. Data are representative of two different tracheal rings.

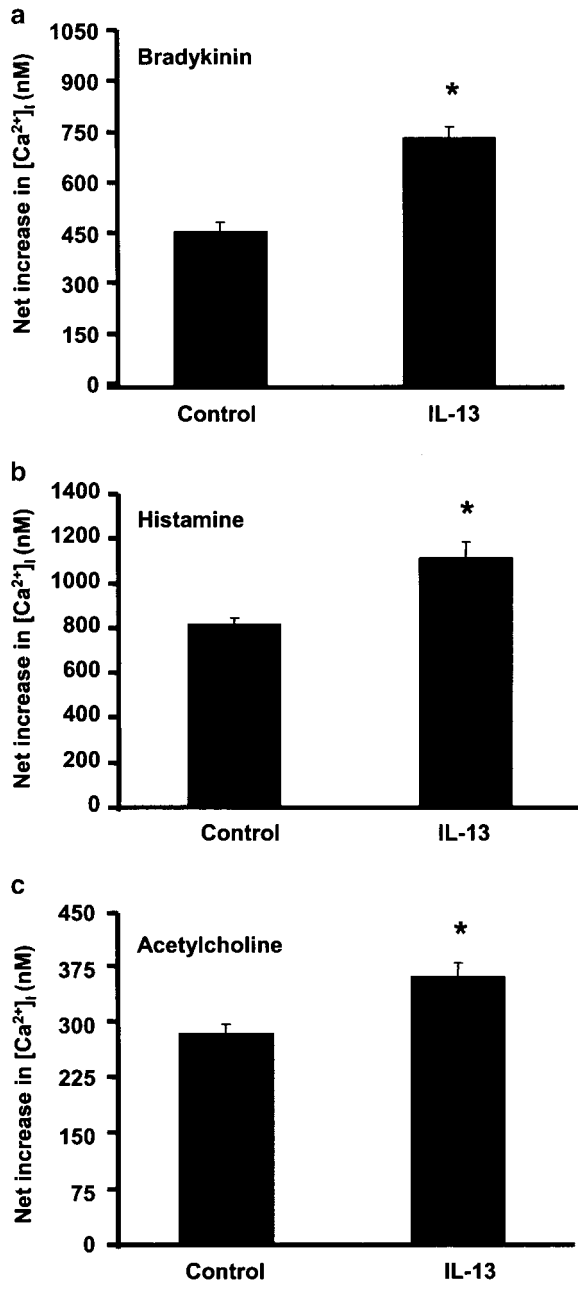

Figure 2 IL-13 enhances agonist-evoked calcium signals in cultured ASM cells. Human ASM cells treated with IL-13 (50 ng ml^{-1}) for 24 h before calcium responses to 1 nM bradykinin (a), 50 μM histamine (b) and 10 μM acetylcholine (c) were performed in Fura-2-loaded cells. Results are expressed as the net increase in intracellular calcium concentration (in nM) at the peak and values are means $\pm$ s.e.m. *$P < 0.05$, significantly different from cells treated with the diluent alone.

effective concentrations of three different G protein-coupled receptor (GPCR) agonists. In control cells, the net increases in cytosolic-free calcium (in nM) to bradykinin, histamine and acetylcholine were 463 ± 28 ($n = 49$), 818 ± 25 ($n = 113$) and 283 ± 12 ($n = 78$), respectively. Interestingly, calcium responses to agonists were significantly ($P < 0.05$) increased in ASM cells pretreated with 50 ng ml^{-1} IL-13 for 24 h by 60% (bradykinin, $n = 70$), 35% (histamine, $n = 91$) and 26% (acetylcholine, $n = 54$). IL-13 alone has no effect on intracellular calcium levels (data not shown). These data demonstrate that IL-13 enhances GPCR agonist-associated calcium signals in human ASM cells.

Discussion Evidence now suggests that cytokines play an important role in the development of BHR, an exaggerated airway narrowing in response to a variety of stimuli including contractile agonists (Amrani & Panettieri, 2002). Recent studies using animal models of allergic asthma demonstrate that IL-13, a Th2-type cytokine, plays a major role in the regulation of BHR (Grunig *et al*., 1998; Wills-Karp *et al*., 1998; Walter *et al*., 2001; Akbari *et al*., 2003). In agreement with Grunstein *et al*. (2002), our study shows that IL-13 has the potential to increase the contractile responses to acetylcholine and KCl using an *ex vivo* model of airway reactivity (Chen *et al*., 2003) that expresses both IL-13 receptors, IL-13Rα1 and IL-13Rα2 (Figure 1c). Our study also supports the emerging evidence that IL-13 promotes BHR by directly modulating the function of resident airway cells, although the nature of the cell types remains unknown. A similar conclusion may be drawn from *in vivo* studies showing that IL-13 delivered directly to the airway, either intratracheally or intranasally, generates a BHR to muscarinic receptor stimulation (Kibe *et al*., 2003; Vargaftig & Singer, 2003). It is interesting to note that the induction of BHR by IL-13 even occurred in the absence of any sign of airway inflammation, such as inflammatory cell recruitment, cytokine production and mucus production (Yang *et al*., 2001; Venkayya *et al*., 2002). This inflammation-independent component of BHR by IL-13 may involve a direct effect on ASM, an essential effector tissue that regulates the bronchomotor tone (Amrani & Panettieri, 2003). Owing to the central role of intracellular calcium in regulating ASM contractility (Amrani & Panettieri, 2002), we investigated the effect of an effective concentration of IL-13 on the calcium signals generated by contractile agonists in cultured ASM cells that were shown to express both IL-13 receptors (Laporte *et al*., 2001). In a nonspecific manner, IL-13 potentiates the calcium responses induced by three different contractile agonists, supporting the novel hypothesis that IL-13 may increase ASM contractility and possibly BHR previously described in animal models of allergic asthma by altering calcium homeostasis in ASM cells. The ability of IL-13 to increase ASM responsiveness induced by high concentrations of KCl (100 mM) suggests the possible involvement of calcium-independent pathways. In that regard, IL-13 may increase ASM contractility by modulating the contractile machinery, either by enhancing the sensitivity of the myofilaments to calcium or by rearranging the cytoskeleton apparatus as previously shown with TNFα in human ASM cells (Hunter *et al*., 2003). As IL-13 also regulates the expression of various proinflammatory genes in ASM cells (Laporte *et al*., 2001; Hirst *et al*., 2002), collectively these data suggest that the interaction of IL-13 with ASM may play an important role in the pathogenesis of asthma.

We thank Mary McNichol for assistance in the preparation of the manuscript. This work was supported by NIH Grants 2R01-HL55301 (RAP), 1P50-HL67663 (RAP), HL057498 (MSK) and by an American Lung Association Grant RG-062-N (YA). Yassine Amrani is a Parker B. Francis Fellow in Pulmonary Research.

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(Received August 12, 2003
Revised September 16, 2003
Accepted September 24, 2003)