

Interleukin-4 increases murine airway response to kinins, via up-regulation of bradykinin B₁-receptors and altered signalling along mitogen-activated protein kinase pathways

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Summary

Background IL-4 is believed to play a role in asthma and chronic obstructive pulmonary disease through promotion of eosinophilic inflammation and mucus hypersecretion. Whether IL-4 can induce a direct effect on airway smooth muscle remains unknown.

Objective To investigate the effect of IL-4 on airway smooth muscle, focusing on the contractile response to des-Arg⁹-bradykinin and bradykinin.

Methods Tracheal segments from murine airways were cultured for 1–8 days in the absence and presence of IL-4. The smooth muscle response induced by des-Arg⁹-bradykinin and bradykinin was investigated in myographs. Expression levels for the IL-4-, bradykinin B₁- and B₂-receptors were characterized using RT-PCR. Specific inhibitors were used to study signal changes along the IL-4 receptor- (IL-4R-) coupled mitogen-activated protein (MAP) kinase (MAPK) pathways.

Results IL-4 treatment increased the contractile response to des-Arg⁹-bradykinin and bradykinin in a concentration- and time-dependent manner. Dexamethasone and the transcriptional inhibitor actinomycin D blocked this effect. c-Jun N-terminal kinase inhibitor SP600125 also blocked the effect of both des-Arg⁹-bradykinin and bradykinin, whereas p38 inhibitor SB203580 blocked only the former and the MAPKK inhibitor PD098059, only the latter agonist responses. IL-4 treatment increased the mRNA levels representing bradykinin B₁- but not B₂-receptors. Levels of IL-4R were not altered during culture.

Conclusion Long-term exposure to IL-4 increases the contractile response induced by des-Arg⁹-bradykinin and bradykinin in cultured murine airways. This effect appears to be mediated via an up-regulation of B₁-receptors and altered signalling along the MAPK pathways.

Keywords airway smooth muscle cell, bradykinin, IL-4

Submitted 27 August 2003; revised 6 April 2004; accepted 28 May 2004

Introduction

IL-4 is critically important for the development of allergic inflammation [1]. It is believed to play a role in asthma and chronic obstructive pulmonary disease (COPD) through promotion of eosinophilic inflammation by increasing eotaxin expression and inhibiting eosinophils apoptosis [2]. In addition, IL-4 induces mucus hypersecretion that contributes to airway obstruction [3]. Whether IL-4 can induce a direct effect on airway smooth muscle remains unknown. However, airway smooth muscle cells have a plastic phenotype and can change their appearance as a result of stimulation [4]. Chronic inflammation established during asthma may alter their functional properties, which might interfere with the development of airway hyper-reactivity. The primary target of the present study was to investigate if isolated airways, exposed to IL-4 during prolonged periods, change their response to

contractile agonists. To this end, responses to bradykinin with and without IL-4 pre-treatment, were investigated in a novel organ culture assay, designed to study the endured effects of inflammatory mediators on airway smooth muscle [5].

Bradykinin is a peptide, which is produced in the blood or tissues during inflammation. It can be cleaved into des-Arg⁹-bradykinin [6]. Both analogues act by stimulating distinct receptors, B₁ and B₂. Bradykinin exerts most of its actions through binding of B₂-receptors while des-Arg⁹-bradykinin binds to B₁-receptors. The B₂-receptor is often constitutively expressed in smooth muscle and epithelial cells while the B₁-receptor is induced during inflammation [7]. Bradykinin produce a number of effects which are relevant for airway pathophysiology [8]; bronchoconstriction, plasma protein extravasation, mucus secretion and stimulation of inflammatory cells. A secondary target in the present investigation was to investigate if the expression of bradykinin receptors on airway smooth muscle could be affected by IL-4 treatment.

IL-4 exerts its effects through binding of the IL-4 receptor (IL-4R) [9, 10]. The IL-4R is one of several ways to induce activation along the mitogen-activated protein (MAP) kinase

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(MAPK) signal transduction pathways. These pathways have recently emerged as some of the more important intracellular signalling networks involved in pulmonary pathophysiology [11]. The final target of the present study was to investigate the signal transduction pathways for IL-4 that are involved in alterations of bradykinin responses.

Materials and methods

Tissue preparation and organ culture

Male BALB/c J strain mice, 8–10 weeks old (Taconic M&B A/S, Ry, Denmark) were killed by cervical dislocation. Whole tracheae were rapidly removed and placed into Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL). Under sterile conditions each trachea was dissected free from adherent tissue and cut into segments containing three cartilage rings each. Segments were used directly or placed individually into wells of a 96-well plate (Ultra-low attachment; Sigma, St Louis, MO, USA) with 300 µL DMEM for organ culture. Incubation was performed at 37 °C in humidified 5% CO₂ in air for 1, 2, 4 or 8 days in the absence or presence of IL-4 (10, 100 or 300 ng/mL). Everyday segments were moved into a new well containing fresh media. In order to study the effects of actinomycin D and dexamethasone on des-Arg⁹-bradykinin- and bradykinin-induced contractions, segments were cultured for 1 day with IL-4 (100 ng/mL) in the absence or presence of either actinomycin D (5 µg/mL) or dexamethasone (1 µM). To study intracellular mechanisms segments were incubated with IL-4 (100 ng/mL) and the c-Jun N-terminal kinase (JNK) inhibitor SP600125, MAP kinase MEK 1/2 inhibitor PD098059 or p38 inhibitor SB203580 (1 or 10 µM) for 4 days. Control segments received the same amount of IL-4 and in addition dimethyl sulphoxide (DMSO) as vehicle since SP600125, PD098059 and SB203580 were solved in DMSO. After incubation, des-Arg⁹-bradykinin- and bradykinin-induced concentration–effect curves on the segments were obtained. In order to evaluate whether IL-4 and dexamethasone affect transcription of the IL-4R and B₁- and B₂-receptors, whole tracheae were placed into wells of a 24-well plate containing 1 mL DMEM and incubated in 37 °C for 4 days in the absence or presence of IL-4 and dexamethasone (100 ng/mL and 1 µM, respectively). The smooth muscle strips were then isolated mechanically on an ice tray under a microscope. After removal of tracheal epithelium and cartilages, the smooth muscle strips were stored in RNAlater™ (QIAGEN GmbH, Hilden, Germany) at –80 °C until extraction of total RNA.

Isometric tension measurements

Tracheal smooth muscle reactivity was recorded in temperature-controlled (37 °C) myographs (Organ Bath Model 700MO, J.P. Trading, Denmark) containing 5 mL Krebs–Henseleit buffer with 10 mM D-glucose. The solution was continuously equilibrated with 5% CO₂ in O₂ resulting in a stable pH of 7.4. The tracheal segments were mounted on two L-shaped metal tacks. One of the tacks was connected to a force–displacement transducer for continuous recording of isometric tension by the Chart software (AD Instruments

Ltd, Hastings, UK). The other tack was connected to a displacement device in order to be able to change the distance between the two tacks and thus the tension in the mounted segments. One-hour equilibration was performed by washing every 15 min and adjusting the basal tension to 0.8 mN [5]. Each segment was contracted with 60 mM KCl to test their contractile function. To inhibit epithelial prostaglandin release, the segments were incubated with 3 µM of the cyclooxygenase-inhibitor indomethacin 50 min before increasing concentrations of des-Arg⁹-bradykinin or bradykinin were administered. In addition, to exclude that cholinergic nerve activation affected the responses to des-Arg⁹-bradykinin and bradykinin, 5 µM of the muscarinic receptor antagonist atropine was added 50 min before bradykinin or des-Arg⁹-bradykinin. At the end of each experiment, a single contractile response of 1 mM carbachol was induced.

RNA extraction and RT-PCR

Tracheal smooth muscle strips were homogenized and the total RNA was extracted using the RNeasy Mini Kit following the kit instructions (QIAGEN GmbH). The purity of total RNA was measured by a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 in all preparations. Reverse transcription of total RNA to cDNA was carried out using Omniscript™ reverse transcriptase kit (QIAGEN GmbH) in the Mastercycler personal PCR machine (Eppendorf AG, Hamburg, Germany). The RNA was incubated at 65 °C for 5 min to denature. After denaturation, the RNA was immediately cooled and the reverse transcription master mix was added into the tube. The reaction of reverse transcription was carried out at 37 °C for 1 h.

RT-PCR

The real-time quantitative PCR was performed using Light-Cycler FastStart DNA Master SYBR® Green 1 kit (Roche Diagnostics GmbH, Mannheim, Germany) in the Light-Cycler detection system (Roche Diagnostics). The system automatically monitors the binding of the fluorescent dye SYBR Green to double-stranded DNA by real-time detection of the fluorescence during each cycle of PCR amplification. RT-PCR was performed in 20 µL reaction volume and carried out with heating 95 °C for 10 min followed by 45 PCR cycles of 95 °C for 10 s, 55 °C for 5 s (initial 65 °C, followed by a 2 °C decrease of the first five cycles) and 72 °C for 5 s. Thereafter, melting curve analysis was made (65–95 °C) in order to verify that only one product was amplified. Samples with more than one peak were excluded. The data were analysed with comparative cycle threshold (C_T) method.

All PCR primers used in the present study were designed by using Prime Express® 2.0 software (Applied Biosystem, Foster City, CA, USA) and synthesized by DNA Technology A/S (Aarhus, Denmark). Below are the sequences:

IL-4Rα fwd: 5'-GAATAGGCCCGGCCGGTCCAATCAG-3'
 IL-4Rα rev: 5'-TGTGAGGTTGTCTGGAGCTAGG-3'
 B₁-receptor fwd: 5'-CGTTCAACTCCACCATCCTTACA-3'
 B₁-receptor rev: 5'-CCTTCTGCGTTCCGTCAAAG-3'
 B₂-receptor fwd: 5'-CTTTGTCCGGCGTCCAAA-3'
 B₂-receptor rev: 5'-GACTTGTGTGGTGACGTTGAACA-3'

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
 fwd: 5'-CATGGCCTTCCGTGTTCTTA-3'
 GAPDH rev: 5'-TGCTTACCACCTTCTTGATG-3'.

Analysis

Functional data are expressed as mean values $\pm$ SEM. Responses of des-Arg⁹-bradykinin and bradykinin are presented as percentage of contraction to 1 mM carbachol. This concentration of carbachol has been shown to induce a contraction close to the maximal capacity of both fresh and cultured segments [5]. Agonist concentration–effect curves were fitted to the Hill equation using an iterative, least-square method (GraphPad Prism), to provide estimates of maximal contraction ($E_{\max}$) and midpoint location (pEC_{50}). Statistical analysis was performed using unpaired *t*-test, one-way ANOVA with Dunnnett's or Bonferroni post-test and two-way ANOVA with multiple comparisons. $P \leq 0.05$ was considered to be significant.

Results

Contractile effect of carbachol on isolated mouse tracheal segments

The contraction induced by a high concentration of carbachol (1 mM) at the end of the experiments was used as a reference in all myograph experiments. There was no significant difference in the response between fresh segments and segments cultured for 1, 2, 4 and 8 days. Moreover, no differences were obtained in segments treated with IL-4 (Fig. 1).

Contractile effect of des-Arg⁹-bradykinin and bradykinin on mouse tracheal segments, fresh and cultured for 4 days with different concentrations of IL-4

Contractile responses to des-Arg⁹-bradykinin and bradykinin were studied in freshly isolated segments. Compared with contraction induced by carbachol (9.68 ± 0.90 mN; $n = 6$) the responses to des-Arg⁹-bradykinin and bradykinin were negligible (Table 1). In order to examine the effect of IL-4 on the kinin-induced contractions, the responses of the tracheal segments were studied after 4 days of culture with different concentrations of IL-4 (10, 100 and 300 ng/mL). Compared with control segments, which were cultured in absence of IL-4, there was an increase in the contractile response to des-Arg⁹-bradykinin for segments cultured with

100 ng/mL of IL-4 (Table 1). No further increase was obtained with 300 ng/mL. Treatment with 100 and 300 ng/mL but not 10 ng/mL of IL-4 increased the contractile response to bradykinin compared with control segments (Table 1). The higher concentration of 300 ng/mL IL-4 did not cause any further increase of the bradykinin contraction.

Contractile effect of des-Arg⁹-bradykinin and bradykinin on mouse tracheal segments cultured for 1, 2, 4 and 8 days in the absence or presence of IL-4

To investigate if the effect of IL-4 was time dependent, the contractile responses of tracheal segments to des-Arg⁹-bradykinin and bradykinin were studied after 1, 2, 4 and 8 days of culture in the absence or presence of 100 ng/mL IL-4. All segments cultured for 1, 2, 4 or 8 days with IL-4 demonstrated an increased contractile response to des-Arg⁹-bradykinin and bradykinin compared with control segments cultured without IL-4 for the same time period (Table 1). The response to des-Arg⁹-bradykinin increased after 1 and 2 days of culture but did not increase more after 4 and 8 days of culture (Fig. 2). The response to bradykinin increased after 1, 2 and 4 days of culture but there was no further increase after 8 days of culture (Fig. 3). A tendency of an increased potency for des-Arg⁹-bradykinin was demonstrated, but significantly only after 1 day of culture. Since bradykinin induced such low contractions in cultured control segments, any changes of the potency in IL-4-treated segments could not be determined (Table 1).

Effects of dexamethasone on the contractile effect of des-Arg⁹-bradykinin and bradykinin on mouse tracheal segments cultured for 1 day with IL-4

In order to investigate if the anti-inflammatory substance dexamethasone could affect the IL-4-induced increase of the contractile response to des-Arg⁹-bradykinin and bradykinin segments were cultured with IL-4 in the absence or presence of dexamethasone for 1 day. Segments cultured in the presence of dexamethasone demonstrated a low response to both des-Arg⁹-bradykinin and bradykinin ($8.0 \pm 3.4\%$, respectively, $4.9 \pm 2.2\%$) compared with control segments that were cultured without dexamethasone ($20.4 \pm 4.3\%$, respectively, $22.4 \pm 2.9\%$) (Fig. 4).

Effects of actinomycin D on the contractile effect of des-Arg⁹-bradykinin and bradykinin on mouse tracheal segments cultured for 1 day with IL-4

Mouse tracheal segments were cultured for 1 day with IL-4 in the absence or presence of the transcriptional inhibitor actinomycin D in order to see if the IL-4-induced increase of the contractile responses to des-Arg⁹-bradykinin and bradykinin was dependent on transcription. Segments cultured with actinomycin D elicited negligible responses to both des-Arg⁹-bradykinin and bradykinin ($1.4 \pm 0.7\%$, respectively, $2.1 \pm 1.0\%$) compared with control segments cultured without actinomycin D ($20.4 \pm 4.3\%$, respectively, $22.4 \pm 2.9\%$) (Fig. 4).

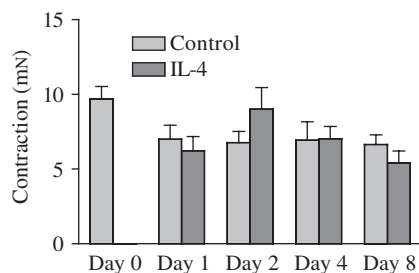
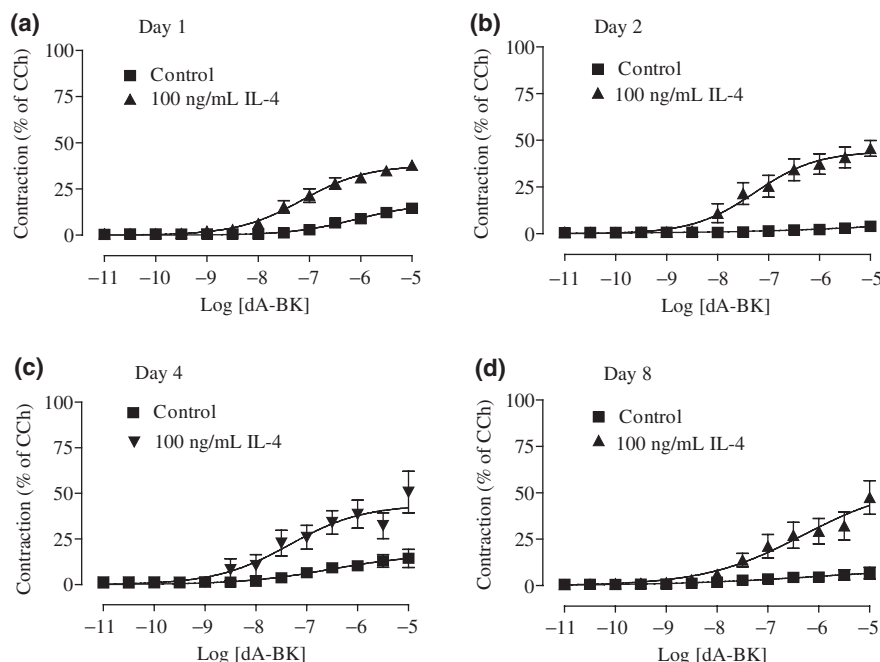


Fig. 1. Carbachol-induced responses of mouse tracheal segments, fresh and cultured for 1, 2, 4 and 8 days in the absence or presence of IL-4. Results are expressed as the mean $\pm$ SEM of six segments.

Table 1. Contractile responses to des-Arg⁹-bradykinin and bradykinin in mouse tracheal segments, fresh and following culture for 1, 2, 4 and 8 days in absence or presence of IL-4

			Des-Arg ⁹ -bradykinin				Bradykinin		
			<i>E</i> _{max}			<i>E</i> _{max}			
Incubation time	IL-4 (ng/mL)	<i>n</i>	% of CCh	mN	pEC ₅₀	<i>n</i>	% of CCh	mN	pEC ₅₀
Day 0	0	6	0.6 ± 0.3	0.1 ± 0.0	ND	6	0.7 ± 0.2	0.1 ± 0.0	ND
Day 1	0	6	17.7 ± 1.3	1.2 ± 0.2	6.3 ± 0.2	7	8.6 ± 1.3	0.8 ± 0.2	ND
	100	8	38.1 ± 1.8*	2.4 ± 0.4*	7.1 ± 0.2*	5	39.5 ± 4.1*	4.5 ± 0.7*	6.0 ± 0.2
Day 2	0	5	4.1 ± 1.4	0.7 ± 0.3	ND	7	14.2 ± 4.4	0.9 ± 0.3	ND
	100	6	47.1 ± 2.7*	4.4 ± 0.6*	7.2 ± 0.4	5	46.6 ± 4.4*	3.3 ± 1.0*	7.7 ± 0.3
Day 4	0	6	21.1 ± 4.7	1.5 ± 0.3	6.7 ± 0.1	6	18.9 ± 6.3	1.3 ± 0.4	ND
	10	7	20.0 ± 5.9	1.2 ± 0.4	6.9 ± 0.2	6	19.1 ± 7.6	1.5 ± 0.7	ND
	100	5	44.0 ± 6.6*	2.8 ± 0.4*	7.6 ± 0.3	8	63.4 ± 7.4*	4.4 ± 0.7*	7.8 ± 0.2
Day 8	300	6	37.9 ± 10.7	2.4 ± 0.6	6.5 ± 0.4	6	48.1 ± 8.0*	3.4 ± 0.5	7.6 ± 0.5
	0	8	6.8 ± 3.1	0.4 ± 0.2	ND	8	5.4 ± 1.9	0.4 ± 0.1	ND
	100	5	36.1 ± 11.3*	2.0 ± 1.0	7.1 ± 0.1	5	58.0 ± 8.8*	3.7 ± 0.9*	7.4 ± 0.2

CCh, carbachol; ND, not determined.

Statistical analysis was made using unpaired *t*-test and one-way ANOVA with Dunnett's post-test.**P* < 0.05.**Fig. 2.** The contractile response to des-Arg⁹-bradykinin (dA-BK) of mouse tracheal segments cultured for 1 (a), 2 (b), 4 (c) and 8 days (d) in the absence or presence of IL-4. Each point represents the mean of all segments tested with error bars representing SEM (*n* = 6–8).

Effects of specific MAPK inhibitors: JNK inhibitor SP600125, MAPKK MEK 1/2 inhibitor PD098059 and p38 inhibitor SB203580

To study if IL-4 activates the JNK, extracellular signal-related kinase (ERK) and p38 pathways tracheal segments were cultured for 4 days with IL-4 (100 ng/mL) in the presence of 1 or 10 μ M of SP600125, PD098059 or SB203580. After culture, the contractile responses to des-Arg⁹-bradykinin and bradykinin were analysed. Segments cultured with 10 μ M of SP600125 elicited a decreased response to both des-Arg⁹-bradykinin (19.0 ± 7.7%) and bradykinin (25.9 ± 9.5%) compared with control segments (59.8 ± 1.5%, respectively, 69.8 ± 1.7%) and segments cultured with 1 μ M of SP600125 (58.2 ± 2.2%,

respectively, 65.5 ± 1.2%) (Figs 5a and b). Segments cultured with 1 and 10 μ M of PD098059 did not demonstrate a decreased response to des-Arg⁹-bradykinin (35.9 ± 6.5%, respectively, 46.6 ± 5.5%) compared with control segments (47.4 ± 4.7%) (Fig. 5c). Culture with 10 but not 1 μ M of PD098059 resulted in a decreased response to bradykinin (26.0 ± 8.5%, respectively, 44.2 ± 7.6%) compared with control segments (59.0 ± 3.8%) (Fig. 5d). Segments cultured with 10 μ M of SB203580 demonstrated a decreased response to des-Arg⁹-bradykinin (48.9 ± 3.1%) compared with control segments (60.9 ± 2.3%) and segments cultured with 1 μ M of SB203580 (65.7 ± 2.1%) (Fig. 5e). Culture with 1 and 10 μ M of SB203580 did not decrease the response to bradykinin (65.9 ± 2.0%, respectively, 64.0 ± 2.3%) compared with

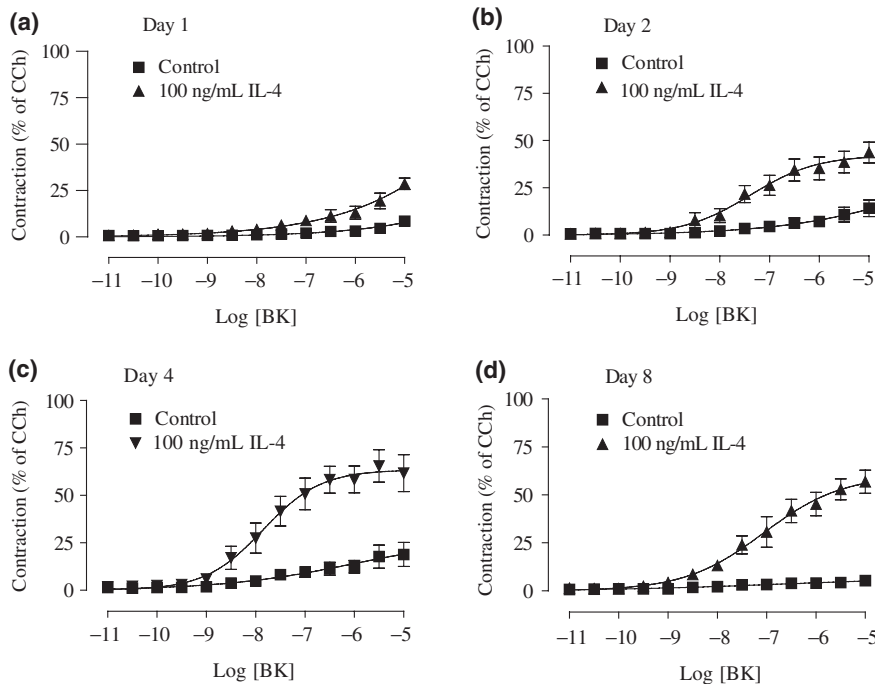


Fig. 3. The contractile response to bradykinin (BK) of mouse tracheal segments cultured for 1 (a), 2 (b), 4 (c) and 8 days (d) in the absence or presence of IL-4. Each point represents the mean of all segments tested with error bars representing SEM ($n = 6-8$).

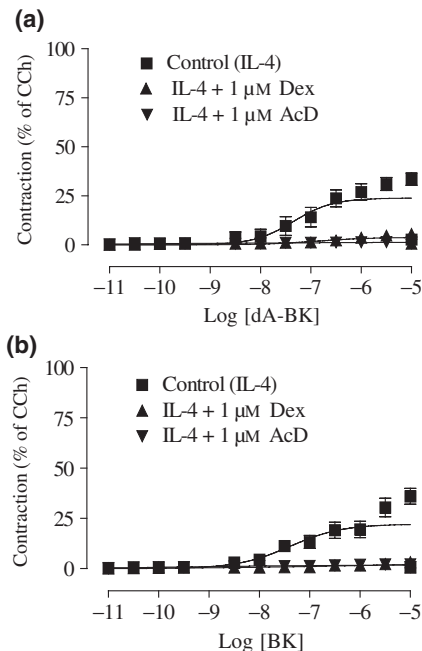


Fig. 4. The contractile responses to des-Arg⁹-bradykinin (dA-BK) (a), and bradykinin (BK), (b), after 1 day of culture with IL-4 and in the absence or presence of either dexamethasone (Dex) or actinomycin D (AcD). Each point represents the mean of all segments tested with error bars representing SEM ($n = 6-8$).

control segments ($65.8 \pm 2.1\%$) (Fig. 5f). In order to evaluate if the IL-4 induced increases occurs through the same mechanism as the basal responses segments were cultured (4 days) in the absence or presence of $10 \mu\text{M}$ SP600125, SB203580 or PD098059, respectively. After 4 days of culture control segments (cultured with DMSO only) elicited a contractile response to bradykinin ($36.5 \pm 5.6\%$) but the response to

des-Arg⁹-bradykinin ($5.8 \pm 2.1\%$) was almost negligible. Response to bradykinin was significantly decreased in segments cultured with $10 \mu\text{M}$ of PD098059 ($15.6 \pm 6.7\%$) and SP600125 ($9.2 \pm 2.7\%$), but not SB203580 ($22.7 \pm 4.2\%$) (Fig. 5g, h).

Effects of IL-4 and dexamethasone on the expression of IL-4R α , B₁- and B₂-receptors in tracheal smooth muscle cells

Expression of IL-4R α and B₁- and B₂-receptor mRNA in tracheal smooth muscle cells was studied using RT-PCR after 4 days of culture in the absence or presence of IL-4 and dexamethasone. Compared with control segments cultured in absence of IL-4, segments cultured in presence of IL-4 demonstrated a twofold increase in the expression of B₁-receptors, but no significant change in the expression of B₂-receptors and IL-4R α . Segments cultured with both IL-4 and dexamethasone showed a decreased expression of B₂-receptors, but the expression of the IL-4R and B₁-receptor was not altered, compared with control segments (Fig. 6). In order to evaluate the effects of dexamethasone, comparisons between segments cultured with IL-4 alone and segments cultured with IL-4 together with dexamethasone were made. This analysis demonstrated that the effect of IL-4 on B₁-receptor expression was inhibited by dexamethasone ($P < 0.01$).

Discussion

Long-term exposure to IL-4 increased the contractile response induced by bradykinin and des-Arg⁹-bradykinin in cultured murine airways. Treatment with IL-4 induced an up-regulation of the bradykinin B₁-receptor, whereas the B₂-receptor was unaffected. The IL-4 effect appeared to be mediated via the MAPK pathways of signal transduction. Both the B₁- and B₂-receptor responses were increased by JNK activation, whereas activation of ERK only increased

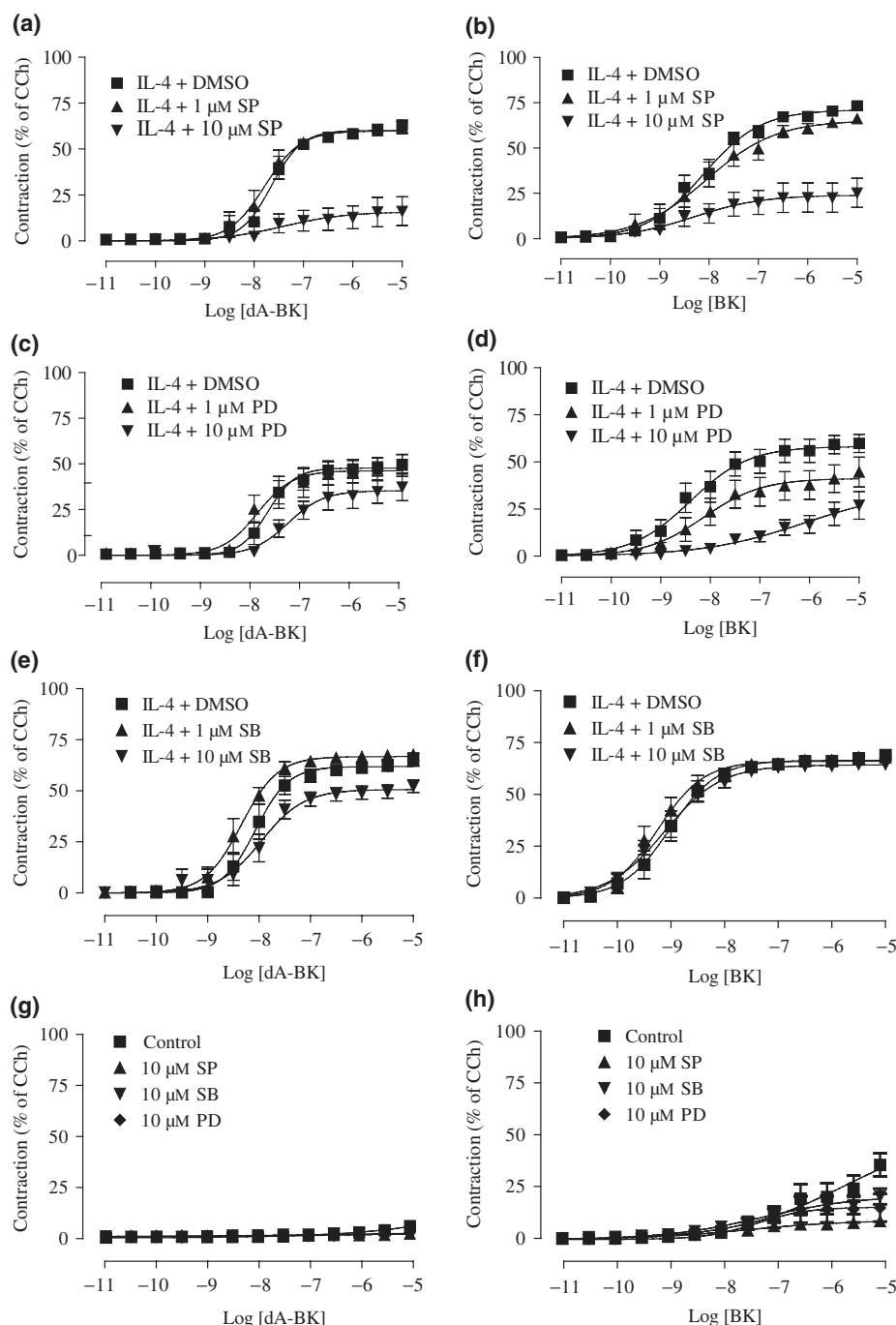


Fig. 5. The contractile responses of mouse tracheal segments to des-Arg³-bradykinin (dA-BK) and bradykinin (BK) after 4 days of culture with IL-4 and different concentrations of either c-Jun N-terminal kinase inhibitor SP600125 (a, b), MAP kinase MEK 1/2 inhibitor PD098059 (c, d) or p38 inhibitor SB203580 (e, f), or after 4 days of culture with only the inhibitors present (g, h). Each point represents the mean of all segments tested with error bars representing SEM ($n = 5-8$).

the B₂-receptor responses and activation of p38 only the B₁-receptor responses.

During normal conditions, both bradykinin and des-Arg⁹-bradykinin are known as poor bronchoconstrictors [12]. This is in accordance with the present results where fresh segments elicited a negligible response to both bradykinin and des-Arg⁹-bradykinin. In contrast to the effect of the two bradykinin receptor agonists, carbachol induced a strong contractile response in fresh segments. Carbachol induces airway contraction via activation of the muscarinic receptor

3, M₃. The M₃ receptor is, like the bradykinin receptors, coupled to the G_q-complex [13].

The contractile responses to des-Arg⁹-bradykinin and bradykinin increased with IL-4, in a concentration-dependent manner. Maximal effect for both agonists was obtained at 100 ng/mL. The effects of IL-4 treatment also appeared to be time dependent. The effects were noted after 1 day of culture and des-Arg⁹-bradykinin and bradykinin reached their maximum after 2 and 4 days of culture, respectively. The effect remained at a maximal plateau during at least 8 days of culture.

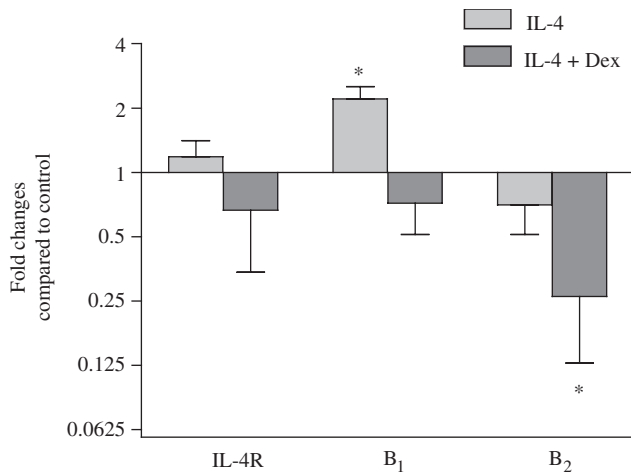


Fig. 6. RT-PCR results after 4 days of culture in the absence or presence of IL-4 and dexamethasone (Dex); $n = 3$. * $P < 0.05$.

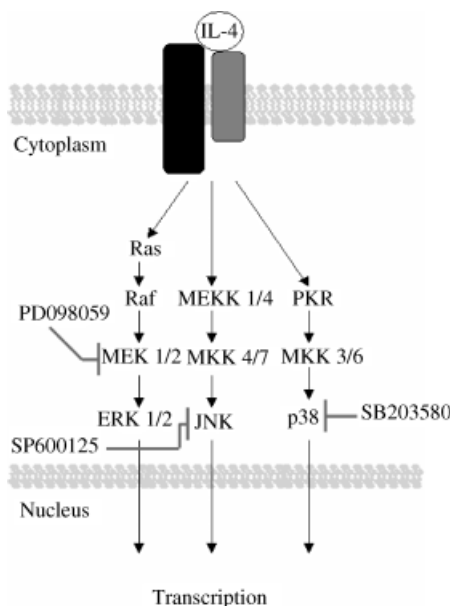


Fig. 7. IL-4 binds to the IL-4R and activates different mitogen-activated protein kinase signal pathways. The major mediators of transduction are extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, here shown in conjunction with their respective inhibitors presently used (PD098059, SP600125 and SB203580). The JNK pathway is involved in regulation of the response to both des-Arg⁹-bradykinin and bradykinin. The ERK pathway only seems to be involved in regulation of the response to bradykinin, while signalling through the p38 pathway only seems to be involved in regulating the response to des-Arg⁹-bradykinin. Illustration detailed in accordance with reference [9,11,14–16].

IL-4 did not affect the contractile response of carbachol, regardless of concentration and culture time.

IL-4 binds to the IL-4R and activates a network of signalling pathways, involving a series of MAPK [9] depicted in Fig. 7. Treatment with JNK inhibitor SP600125 decreased the effect of IL-4 on the response to both des-Arg⁹-bradykinin and bradykinin. PD098059 decreased the effect of IL-4 on the response to bradykinin but not des-Arg⁹-bradykinin, while SB203580 decreased the response to des-Arg⁹-bradykinin but not bradykinin. Altogether, the presented data suggest that IL-4 activates the JNK and ERK pathways in order to

increase the response to bradykinin, whereas JNK and p38 pathways are involved in the response to des-Arg⁹-bradykinin. This is an interesting difference, especially since specific MAPK inhibitors have demonstrated activity in asthma and COPD, providing support for the therapeutic potential of this class of kinase inhibitors in chronic airway disease [11]. The basal response to bradykinin after 4 days of culture was inhibited in segments cultured with 10 μ M SP600125 or PD098059. A possible explanation for this effect might be that the tissue preparation *per se* causes some minor damage to the dissected segments, activating inflammatory pathways similar to the ones induced by IL-4. Protein kinase inhibitors used in this study were chosen because of their reported specificity [14–16].

Treatment with the transcriptional inhibitor actinomycin D blocked the effect of IL-4, indicating that protein transcription is necessary in order to increase the response to des-Arg⁹-bradykinin and bradykinin. In analogy, segments cultured with IL-4 together with dexamethasone, a corticosteroid with anti-inflammatory effects known to affect protein transcription, elicited an almost negligible response to both des-Arg⁹-bradykinin and bradykinin. Thus, these data indicate that dexamethasone inhibits transcription for all three MAPK pathways.

RT-PCR was used to study the expression levels of mRNA for IL-4R, B₁- and B₂- receptors in tracheal smooth muscle cells after 4 days of culture in the absence or presence of IL-4 and dexamethasone. The IL-4R was present during the whole culture period despite treatment with either IL-4 or dexamethasone. Thus, activation of IL-4R can be achieved during all experiment circumstances tested. The twofold increase in the expression levels of B₁-receptors in segments treated with IL-4 indicates that the increased response to des-Arg⁹-bradykinin in conjunction with IL-4 treatment, at least partly, is due to an up-regulation of the B₁-receptor. This effect was not seen in segments treated with a combination of IL-4 and dexamethasone, which is in accordance with the functional experiments. Expression levels of B₂-receptors were not affected by the IL-4 treatment, suggesting that the increased response to bradykinin is not due to an up-regulation of B₂-receptors. However, the B₂-receptor expression was decreased in segments cultured with IL-4 in conjunction with dexamethasone. Apparently, anti-inflammatory glucocorticoids like dexamethasone might act by preventing mRNA expression for both bradykinin receptor subtypes [17].

Treatment with IL-4 increased the contractile response to des-Arg⁹-bradykinin and bradykinin in a concentration- and time-dependent manner, whereas the contractile response to carbachol was not affected. This demonstrates that although all three receptors are G_q-coupled, they may use different signalling pathways. There are different possibilities for how IL-4 induces the increased response to des-Arg⁹-bradykinin and bradykinin. For des-Arg⁹-bradykinin, the up-regulation of B₁-receptors is probably an important factor. However, since signalling through G-protein coupled receptors involves multiple components, there are several other alternatives for how IL-4 might alter bradykinin receptor signalling. G-protein-coupled receptor components are held in close association with each other in different compartments in the lipid bilayer [18]. This compartmentation is believed to make the

signalling more efficient. Bradykinin receptors are situated in small invaginations in the cell membrane called caveolae [19]. Treatment with IL-4 might change the appearance or placement of these caveolae and thereby redistribute the receptors. Such a redistribution could result in an increase of the receptor signalling. Other possibilities for the increased response for B₂-receptors might be decreased phosphorylation and internalization of the receptor or increased translation of the receptor.

The primary conclusion of this study is that IL-4 might induce a direct effect on airway smooth muscle contraction. Since IL-4 is suggested to be involved in the development of asthma, it is possible that IL-4 also *in vivo* acts directly on the airway smooth muscle cells, thereby affecting their response to constrictor agonists [20]. In addition to IL-4, there are several other cytokines involved in the development of asthma and some of them are also able to induce the B₁-receptor. For example, both IL-1 β , TNF- α and IL-8 have been reported to up-regulate the B₁-receptor expression in human lung fibroblasts [21–23]. IL-4 also has effects on expression of receptors other than bradykinin receptors. The expression of neurokinin receptors on murine bone marrow-derived mast cells is increased after culture with IL-4, which increases the sensitivity to substance P [24]. In a similar way, IL-4 might act in concert with bradykinin contributing to airway hyper-reactivity seen in asthma.

Acknowledgements

The present work was supported by the Swedish Medical Research Council, the Swedish Heart Lung Foundation, the Swedish Association for Allergology, the Swedish Foundation for Health Care Science and Allergic Research and the Royal Physiographic Society.

We thank Ingegerd Larsson and Ann Reutherborg for generous and qualified help with the laboratory work.

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