Interleukin-1 β enhances bradykinin-induced phosphoinositide hydrolysis and Ca²⁺ mobilization in canine tracheal smooth-muscle cells: involvement of the Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/MAPK pathway

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Elevated levels of several cytokines including interleukin-1 β (IL-1 β) have been detected in airway fluid of asthmatic patients. Inhalation of IL-1 β induced a bronchial hyper-reactivity to contractile agonists. However, the implication of IL-1 β in the pathogenesis of bronchial hyper-reactivity is not completely understood. Therefore, we investigated the effect of IL-1 β on bradykinin (BK)-induced inositol phosphate [Ins(X)P] accumulation and Ca²⁺ mobilization, and up-regulation of BK receptor density in canine cultured tracheal smooth-muscle cells (TSMCs). Treatment of TSMCs with IL-1 β potentiated BK-induced Ins(X)P accumulation and Ca²⁺ mobilization. However, there was no effect on the Ins(X)P response induced by endothelin-1, 5-hydroxytryptamine or carbachol. Treatment with platelet-derived growth factor B-chain homodimer (PDGF-BB) also enhanced the BK-induced Ins(X)P response. These enhancements by IL-1 β and PDGF-BB might be due to an up-regulation of BK B₂ receptor density ($B_{\max}$), since [³H]BK binding to TSMCs was inhibited by the B₂-selective agonist and antagonist, BK and Hoe

140, but not by B₁-selective reagents. The enhancing effects of IL-1 β and PDGF-BB on Ins(X)P accumulation, Ca²⁺ mobilization and $B_{\max}$ were attenuated by PD98059 [an inhibitor of activation of mitogen-activated protein kinase (MAPK) kinase, MEK] and cycloheximide (an inhibitor of protein synthesis), suggesting that IL-1 β may share a common signalling pathway with PDGF-BB via protein synthesis. Furthermore, overexpression of dominant negative mutants, H-Ras-15A and Raf-N4, significantly suppressed the up-regulation of BK receptors induced by IL-1 β , indicating that Ras and Raf may be required for activation of these kinases. These results suggest that the augmentation of BK-induced responses produced by IL-1 β might be, at least in part, mediated through activation of the Ras/Raf/MEK/MAPK pathway in TSMCs.

Key words: cytokine, growth factor, hyper-reactivity, IL-1 β , inositol phosphate.

INTRODUCTION

Asthma is a disease characterized by chronic inflammation, recruitment of pro-inflammatory cells into the airways and airway remodelling. Remodelling of airway structure results in part from smooth-muscle hyperplasia [1] and hypertrophy [2], which may be major contributors to the airway hyper-responsiveness associated with asthma [3,4]. The mechanisms underlying the hyper-responsiveness of the airways to a variety of stimuli are not completely understood. There is increasing evidence that elevated levels of pro-inflammatory cytokines, including interleukin-1 β (IL-1 β), IL-4, tumour necrosis factor- α and transforming growth factor- β , in the bronchoalveolar lavage fluid are present in allergic asthmatic patients [5–9]. During the inflammatory reaction, denuded epithelium and an exposure of oedematous basal membrane to the airspace are seen [10]. This indicates that, in asthma, cytokines such as IL-1 β could therefore have relatively easy access to tracheal smooth-muscle cells (TSMCs) [6], which might have the potential to stimulate hypertrophic and/or hyperplastic changes in the airway wall smooth muscle, linked with airway hyper-responsiveness.

Several studies have demonstrated that IL-1 β displays various biological effects on different cells, including stimulation of cell proliferation [11–13], up-regulation of cellular metabolism and increased expression of several genes encoding for active molecules [13]. It seems likely that, in analogy with the neointimal thickening process in atherosclerosis, IL-1 β derived from inflammatory cells may play an important role in these processes. Moreover, many cytokines and growth factors have been shown to activate several signal-transduction pathways, including mitogen-activated protein kinase (MAPK) and MAPK kinase (MEK) [14]. Utilizing selective expression of dominant negative mutants, Ras, Raf and MEK have been shown to play a key step towards MAPK activation by growth factors such as platelet-derived growth factor (PDGF) [15,16]. Moreover, PDGF has been shown to promote cell proliferation and activate MAPK in TSMCs [17,18]. It has also been shown that these MAPKs are activated by IL-1 β and by a wide variety of cellular stresses [19–21]. These findings further implicate the role of these MAPK pathways as important signalling mechanisms underlying the IL-1 β -induced airway remodelling associated with hyper-reactivity.

Abbreviations used: BK, bradykinin; DMEM, Dulbecco's modified Eagle's medium; ET-1, endothelin-1; F-12, Ham's nutrient mixture F-12; FBS, fetal bovine serum; 5-HT, 5-hydroxytryptamine; IL-1 β , interleukin-1 β ; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PDGF, platelet-derived growth factor; PDGF-BB, PDGF B-chain homodimer; TSMC, tracheal smooth muscle cell; Ins(X)P, inositol phosphate; fura 2/AM, fura 2 acetoxymethyl ester; [Ca²⁺]_i, intracellular [Ca²⁺].

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In addition, IL-1 β has been shown to play an important role in regulating the airway smooth muscle contractility which characterizes the asthmatic phenotype. Inhalational administration of IL-1 β to animals has been shown to enhance bronchial constrictor responsiveness [22,23]. Moreover, treatment of airway smooth-muscle preparations with IL-1 β *in vitro* has been found to reduce isoproterenol-mediated relaxation or potentiate the responses to contractile agonists and specific antigen challenge [24–26]. IL-1 β has also been shown to up-regulate bradykinin (BK) B₂ receptor number in human synovial cells [27] and human bronchial smooth-muscle cells [28]; similar alterations in receptor properties probably occur in the smooth muscle of canine airways. To date, how IL-1 β alters the pharmacological properties of BK receptors in canine TSMCs is largely unknown. Therefore, the detailed mechanisms of IL-1 β action on BK-induced phosphoinositide hydrolysis and Ca²⁺ mobilization, and on expression of BK receptors, were investigated in these cells.

In addressing these questions, experiments were undertaken to investigate the mechanisms underlying the enhancement by IL-1 β of inositol phosphate [Ins(X)P] accumulation and Ca²⁺ mobilization induced by contractile agonists. Our results show that pretreatment of TSMCs with IL-1 β and PDGF B-chain homodimer (PDGF-BB) greatly enhances the BK-induced responses in these cells. These enhancements by IL-1 β and PDGF-BB might be due to an up-regulation of BK B₂ receptor density ($B_{\max}$). Overexpression of dominant negative mutants, H-Ras-15A and Raf-N4, significantly suppressed the up-regulation of BK receptors induced by IL-1 β , indicating that Ras and Raf may be required for activation of these kinases. Collectively, these results provide an example of signal-transduction-mediated modulation of receptor expression induced by IL-1 β through activation of the Ras/Raf/MEK/MAPK pathway in canine TSMCs.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (F-12) medium, OPTI-MEM I medium, Lipofectamine Plus reagent and fetal bovine serum (FBS) were purchased from Gibco-BRL (Gaithersburg, MD, U.S.A.). *myo*-[³H]Ins (18 Ci/mmol), [³H]BK (71 Ci/mmol) and Hybond C membrane were from Amersham (Little Chalfont, Bucks., U.K.). Fura 2 acetoxymethyl ester (fura 2/AM) was from Molecular Probes (Eugene, OR, U.S.A.). PD98059 was from Calbiochem (San Diego, CA, U.S.A.). Recombinant human IL-1 β was from R&D System (Minneapolis, MN, U.S.A.). Recombinant human PDGF-BB was from Pepro Tech (London, U.K.). BCA protein assay kit was from Pierce (Rockford, IL, U.S.A.). Enzymes and other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Animals

Mongrel dogs, 10–20 kg, purchased from a local supplier were used throughout this study. Dogs were housed indoors in the animal facilities with an automatically controlled temperature and light cycle and fed standard laboratory chow and tap water *ad libitum*. Dogs of either sex were anaesthetized with ketamine (20 mg/kg, w/w, intramuscularly) and pentobarbital (30 mg/kg, w/w, intravenously) and the lungs were ventilated mechanically via an orotracheal tube. The tracheas were surgically removed.

Isolation of TSMCs

The TSMCs were isolated according to methods described previously [29–31]. Briefly, the muscle was dissected, minced and

transferred to the dissociation medium containing 0.2% (w/v) collagenase I, 0.01% (w/v) DNase I, 0.01% (w/v) elastase IV and antibiotics in a physiological solution. The released cells were collected and the residual was again digested with fresh enzyme solution for 1 h at 37 °C. The cell number was counted and the suspension diluted with DMEM/F-12 containing 10% (v/v) FBS to a concentration of 2×10^5 cells/ml. The cell suspension was plated on to 24-well (0.5 ml/well), 12-well (1 ml/well) and 6-well (2 ml/well) culture plates containing glass coverslips coated with collagen for the receptor binding assay, and Ins(X)P accumulation and intracellular [Ca²⁺]_i measurement, respectively. Culture medium was changed after 24 h and then every 3 days.

In order to characterize the isolated and cultured TSMCs and to exclude contamination by epithelial cells and fibroblasts, the cells were identified by an indirect immunofluorescent staining method using a monoclonal antibody of light-chain myosin [32]. Over 95% of the cell preparation was found to be composed of smooth-muscle cells.

Accumulation of Ins(X)Ps

The effect of BK on the hydrolysis of phosphoinositide was assayed by monitoring the accumulation of ³H-labelled Ins(X)P as described by Yang et al. [29]. Cultured TSMCs were incubated with 5 μ Ci/ml of *myo*-[2-³H]Ins at 37 °C for 48 h. The cells were treated with IL-1 β or PDGF-BB for the appropriate time during the *myo*-[³H]Ins labelling period. When inhibitors were used, they were added 1 h prior to the application of IL-1 β or PDGF-BB. TSMCs were washed twice with PBS and incubated in Krebs–Henseleit buffer (pH 7.4) containing 117 mM NaCl, 4.7 mM KCl, 1.1 mM MgSO₄, 1.2 mM KH₂PO₄, 20 mM NaHCO₃, 2.4 mM CaCl₂, 1 mM glucose, 20 mM Hepes and 10 mM LiCl at 37 °C for 30 min. After BK was added at the concentration indicated, incubation was continued for another 60 min. Reactions were terminated by the addition of 5% (w/v) perchloric acid followed by sonication and centrifugation at 3000 g for 15 min.

The perchloric acid-soluble supernatants were extracted four times with ether, neutralized with KOH and applied to a column of AG1-X8 (formate form, 100–200 mesh; Bio-Rad, Hercules, CA, U.S.A.). The resin was washed successively with 5 ml of water and 5 ml of 60 mM ammonium formate/5 mM sodium tetraborate to eliminate free *myo*-[³H]Ins and glycerophosphoinositol, respectively. The fraction of total Ins(X)P was eluted with 5 ml of 1 M ammonium formate/0.1 M formic acid. The amount of [³H]Ins(X)P was determined in a radiospectrometer (Beckman LS5000TA, Beckman, Fullerton, CA, U.S.A.).

Measurement of [Ca²⁺]_i

[Ca²⁺]_i was measured in confluent monolayers with the calcium-sensitive dye fura 2/AM as described by Grynkiewicz et al. [33]. Upon confluence, the cells were cultured in DMEM/F-12 with IL-1 β for 24 h before measurements were made. When inhibitors were used, they were added 1 h prior to the application of IL-1 β . The monolayers were covered with 1 ml of DMEM/F-12 with 1% FBS containing 5 μ M fura 2/AM and were incubated at 37 °C for 45 min. At the end of the period, the coverslips were washed twice with physiological buffer solution containing 125 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 2 mM MgCl₂, 0.5 mM NaH₂PO₄, 5 mM NaHCO₃, 10 mM Hepes and 10 mM glucose, pH 7.4. The cells were incubated in PBS for a further 30 min to complete dye de-esterification. The coverslip was inserted into a quartz cuvette at an angle of $\approx 45^\circ$ to the excitation beam and

placed in the temperature-controlled holder of a Hitachi F-4500 spectrofluorometer (Tokyo, Japan). Continuous stirring was achieved with a magnetic stirrer. Fluorescence of Ca²⁺-bound and -unbound fura 2 was measured by rapidly alternating the dual excitation wavelengths between 340 and 380 nm and electronically separating the resultant fluorescence signals at an emission wavelength of 510 nm. The autofluorescence of each monolayer was subtracted from the fluorescence data. The ratios (*R*) of the fluorescence at the two wavelengths were computed and used to calculate changes in [Ca²⁺]_i. The ratios of maximum (*R*_{max}) and minimum (*R*_{min}) fluorescence of fura 2 were determined by adding ionomycin (10 μ M) in the presence of PBS containing 5 mM Ca²⁺ and by adding 5 mM EGTA at pH 8 in Ca²⁺-free PBS, respectively. The *K*_D of fura 2 for Ca²⁺ was assumed to be 224 nM [33].

[³H]BK binding assay

For detecting the effects of IL-1 β and PDGF-BB on BK receptor density or affinity of TSMCs, [³H]BK was used as a radioligand. Binding assays were performed with confluent TSMCs on 24-well culture plates, treated with or without IL-1 β or PDGF-BB treatment in DMEM/F-12 for 24 h prior to the binding experiments, as described previously [31]. When inhibitors were used, they were added 1 h prior to the application of IL-1 β or PDGF-BB. Total receptor density (*B*_{max}) and dissociation constant (*K*_D) were calculated using the GraphPad Prism program (GraphPad, San Diego, CA, U.S.A.), as described previously [31]. Half-maximal inhibitory concentration (IC₅₀) values were calculated from competition experiments by GraphPad Prism. IC₅₀ values were transformed to apparent inhibitory constant (*K*_i) values. Subtype analysis was performed by fitting the competitive inhibition curves with either a one- or a two-binding-site model using an iterative least-squares fit by GraphPad Prism that corrected for occupancy of [³H]BK and with statistical significance established by Fisher's *F* test. Protein concentration was measured by the method of Bradford [34].

Plasmids and transfection

The plasmids encoding H-Ras-15A and Raf-N4 (dominant negative mutants of Ras and Raf-1), cloned into pZIP-NeoSV and pCGN, respectively, were kindly provided by Dr Channing J. Der (Department of Pharmacology, University of North Carolina at Chapel Hill, NC, U.S.A.). All plasmids were prepared by using Qiagen plasmid-DNA preparation kits.

TSMCs were plated at 3×10^5 cells/ml in 24-well culture plates (0.5 ml/well) for 24 h, reaching about 80% confluence. Cells were washed once with PBS and once with serum-free DMEM/F-12, and 0.2 ml of serum-free OPTI-MEM I medium was added to each well. The complex of DNA and Lipofectamine Plus reagent was prepared according to the instructions of the manufacturer (Gibco-BRL). The amount of transfected plasmid was kept constant (2 μ g of H-Ras-15A, Raf-N4, pZIP-NeoSV and pCGN for each well). The complex of DNA and Lipofectamine Plus reagent (0.05 ml) was added to each well, then incubated at 37 °C for 5 h, at that time 0.25 ml of OPTI-MEM I medium containing 20% FBS was added and incubated for 19 h. After 24 h of transfection, the cells were washed twice with PBS and maintained in DMEM/F-12 containing 10% FBS for 48 h. Cells were then washed once with PBS and incubated with serum-free DMEM/F-12, and treated with IL-1 β for 24 h before binding assay.

Analysis of data

Concentration–effect curves were fitted and EC₅₀ values were estimated using GraphPad Prism. Data were expressed as means $\pm$ S.E.M. and analysed with a two-tailed Student's *t* test. *P* < 0.05 was judged to be significant.

RESULTS

Effect of IL-1 β on BK-induced Ins(X)P accumulation

IL-1 β has been shown to stimulate cell proliferation in tracheal smooth muscle, which may be linked to airway hyper-reactivity [11,12]. We therefore examined whether IL-1 β can modulate the Ins(X)P accumulation induced by contractile agonists. TSMCs were pre-labelled with [³H]Ins for 48 h and treated with or without IL-1 β (50 ng/ml) for 24 h during the period of radio-labelling, before addition of agonists. To define the time course by which IL-1 β modulates BK-induced Ins(X)P response, TSMCs were treated with IL-1 β for various times, and then the amount of Ins(X)P accumulation was compared with untreated cells (basal). IL-1 β caused a time-dependent enhancement of BK-induced Ins(X)P accumulation in these cells (Figure 1A). Enhancement of BK-induced Ins(X)P accumulation was not seen until 4 h of treatment, and maximal enhancement apparently occurred after 24 h of treatment with IL-1 β (Figure 1A). In addition, IL-1 β induced a concentration-dependent enhancement of the BK-induced response (Figure 1B). The half-maximal (EC₅₀) and maximal effects of IL-1 β were obtained at concentrations of 1.8 and 50 ng/ml, respectively. Furthermore, the enhancement of Ins(X)P accumulation by pretreatment with IL-1 β was dependent upon the BK concentrations when measured 60 min after exposure to BK (Figure 1C). IL-1 β increased the maximal BK-induced Ins(X)P accumulation, but did not change the basal level (control, 1600 $\pm$ 250 d.p.m./well; IL-1 β , 1570 $\pm$ 330 d.p.m./well) when the cells were treated with IL-1 β for 24 h. The concentration–effect relationship of BK-induced Ins(X)P accumulation was shifted to the left and BK was substantially more effective in TSMCs treated with IL-1 β than in control cells (Figure 1C). The EC₅₀ values of BK-induced Ins(X)P accumulation in control and IL-1 β -treated cells were 16 $\pm$ 9 and 19 $\pm$ 5 nM, respectively.

In experiments to establish whether treatment of TSMCs with IL-1 β also enhanced Ins(X)P accumulation induced by other contractile agonists, under similar conditions as described above, exposure of TSMCs to IL-1 β for 24 h did not potentiate the Ins(X)P responses to endothelin-1 (ET-1, 1 μ M), 5-hydroxytryptamine (5-HT, 100 μ M) and carbachol (100 μ M; results not shown). It is conceivable that IL-1 β selectively potentiated the Ins(X)P accumulation induced by BK. These results suggest that IL-1 β might induce changes in some components unique to the BK pathway in TSMCs.

Effect of IL-1 β on BK-induced [Ca²⁺]_i change

Since IL-1 β increased the magnitude of airway response to contractile agonists [22,23], and it has been well established that generation of InsP₃ leading to an increase of [Ca²⁺]_i is the predominant signalling mechanism which contributes to the contraction of airway smooth muscle [29,30], we evaluated the effect of IL-1 β on the BK-induced increase in [Ca²⁺]_i. As shown in Figure 2, IL-1 β increased the maximal BK-induced rise in [Ca²⁺]_i, but did not change the resting level of [Ca²⁺]_i (255 $\pm$ 19 nM, *n* = 15). The concentration–effect relationship of

Figure 1 Effect of IL-1 β treatment on BK-induced Ins(X)P accumulation in TSMCs

(A) Time-dependence of IL-1 β augmentation of BK-stimulated Ins(X)P accumulation. Cells were pre-labelled with [³H]Ins for 48 h and incubated with either vehicle (basal, no IL-1 β and no BK) or IL-1 β (50 ng/ml) at 37 °C for the indicated times during the [³H]Ins labelling period, and then exposed to BK (10 μ M) for 60 min. (B) Concentration-dependence of IL-1 β augmentation of BK-stimulated Ins(X)P accumulation. [³H]Ins-pre-labelled TSMCs were incubated with either vehicle (no IL-1 β and no BK) or IL-1 β at the concentrations indicated for 24 h during radiolabelling, and then exposed to BK (10 μ M) for 60 min. (C) Effect of IL-1 β treatment on the concentration-effect relationship for BK-induced Ins(X)P accumulation. [³H]Ins-pre-labelled TSMCs were incubated with either vehicle (Control) or IL-1 β (50 ng/ml) for 24 h during radiolabelling, and then exposed to various concentrations of BK for 60 min. The accumulation of Ins(X)P was determined as described in the Materials and methods section. Data are expressed as means $\pm$ S.E.M. from three separate experiments performed in triplicate. * P < 0.05, ** P < 0.01, compared with the control.

the BK-induced increase in [Ca^{2+}]_i was shifted to the left and BK was substantially more effective in TSMCs treated with IL-1 β than in the control cells (Figure 2). The EC₅₀ values of BK-induced Ca^{2+} mobilization in control and IL-1 β -treated cells were 66 ± 13 and 21 ± 5 nM, respectively.

Figure 2 Effect of IL-1 β treatment on the concentration-effect relationship for BK-induced Ca^{2+} mobilization

TSMCs were incubated with either vehicle or IL-1 β (50 ng/ml) for 24 h and then exposed to various concentrations of BK. (A) The cells on glass coverslips were loaded with 5 μ M fura 2/AM and fluorescent measurement of [Ca^{2+}]_i was carried out in a dual excitation wavelength (340 and 380 nm) spectrofluorometer; a, IL-1 β ; b, control. Both cell preparations were exposed to 10 μ M BK. (B) Data are the net increase above the resting level of [Ca^{2+}]_i (270 ± 11 nM) and are expressed as means $\pm$ S.E.M. from six separate experiments. Concentration-effect curves were fitted using using GraphPad Prism.

Table 1 Effects of IL-1 β and PDGF-BB treatment on BK-induced accumulation of Ins(X)P in canine TSMCs

TSMCs were pre-labelled with [³H]Ins for 48 h, treated with IL-1 β (50 ng/ml) or PDGF-BB (20 ng/ml) in the absence or presence of PD98059 (30 μ M) or cycloheximide (1 μ M) for 24 h during the period of radiolabelling, before being exposed to BK (10 μ M) for 60 min. The results are expressed as means $\pm$ S.E.M. from three separate experiments determined in triplicate. * P < 0.01, compared with respective untreated control cells; † P < 0.01, compared with control cells stimulated with IL-1 β or PDGF-BB.

Treatment	Ins(X)P accumulation [(d.p.m./well) $\times 10^{-4}$]	
	Basal	BK
Control		
Untreated	0.26 $\pm$ 0.02	0.91 $\pm$ 0.05
IL-1 β	0.32 $\pm$ 0.03	3.97 $\pm$ 0.21*
PDGF-BB	0.3 $\pm$ 0.02	2.51 $\pm$ 0.19*
PD98059		
Untreated	0.28 $\pm$ 0.02	0.66 $\pm$ 0.05
IL-1 β	0.26 $\pm$ 0.02	1.5 $\pm$ 0.06*†
PDGF-BB	0.32 $\pm$ 0.01	0.41 $\pm$ 0.02†
Cycloheximide		
Untreated	0.18 $\pm$ 0.02	0.66 $\pm$ 0.03
IL-1 β	0.2 $\pm$ 0.01	0.8 $\pm$ 0.03†

Effects of MEK inhibitors on IL-1 β -enhanced responses to BK

To determine whether the enhancing effect of IL-1 β on BK-induced responses was mediated through the activation of the p42/p44 MAPK pathway, which may share this common pathway with growth factors, the effects of IL-1 β and PDGF-BB on BK-induced Ins(X)P accumulation were measured in TSMCs pretreated with PD98059. As shown in Table 1, pretreatment of TSMCs with IL-1 β or PDGF-BB for 24 h enhanced BK-induced Ins(X)P accumulation. Prior treatment of TSMCs with PD98059

Figure 3 Effects of PD98059 and cycloheximide on the potentiating effect of IL-1 β on BK-induced Ca^{2+} mobilization

Canine TSMCs were incubated in the absence (A) or presence (B) of 50 ng/ml IL-1 β for 24 h, in the absence (a) or presence of PD98059 (30 μ M, b) or cycloheximide (CHH, 1 μ M, c). When PD98059 or cycloheximide were used, they were added 1 h prior to the application of IL-1 β . After treatment with IL-1 β , TSMCs were loaded with 5 μ M fura 2/AM and stimulated with 10 μ M BK. The change in $[Ca^{2+}]_i$ was measured as described in the Materials and methods section. (C) Data are the net increase in the transient peak induced by BK above the resting levels and are expressed as means $\pm$ S.E.M. from five experiments. ** $P < 0.01$, compared with control stimulated with IL-1 β .

(30 μ M) significantly attenuated the enhancing effect of IL-1 β or PDGF-BB on the BK-induced Ins(X)P response. In a parallel experiment, treatment of TSMCs grown on collagen-coated glass coverslips with 30 μ M PD98059 significantly attenuated the BK-induced increase in $[Ca^{2+}]_i$, as compared with IL-1 β -treated cells (Figure 3). These results suggest that the enhancing effect of IL-1 β might be mediated through the activation of p42/p44 MAPK in canine TSMCs.

To determine whether the enhancement of BK-induced responses is related to protein(s) synthesis, cycloheximide, an inhibitor of protein synthesis, was used with IL-1 β . Concurrent exposure of TSMCs with cycloheximide blocked the IL-1 β -induced enhancement of Ins(X)P accumulation and Ca^{2+} mobilization in response to BK (Table 1 and Figure 3). In addition, the cytotoxic effects of PD98059 and cycloheximide were evaluated by exclusion of Trypan Blue. Under these experimental conditions, they did not show any toxic effect on TSMCs.

Effect of IL-1 β on [3 H]BK binding

IL-1 β has been shown to enhance the expression of BK receptors in several cell types [28,35,36]. From our study, it is evident that pretreatment of TSMCs with IL-1 β significantly enhances BK-induced accumulation of Ins(X)P and causes an increase in $[Ca^{2+}]_i$. This might be due to changes in the affinity (dissociation constant, K_D) and/or an increase in the density of BK receptors (B_{max}). To determine whether the potentiation by IL-1 β and PDGF-BB on BK-stimulated responses in TSMCs occurs at the level of BK receptors, we further measured B_{max} and K_D in these

Table 2 Effects of IL-1 β and PDGF-BB treatment on [3 H]-BK binding in canine TSMCs

Cultured TSMCs were incubated in the absence or presence of PD98059 (30 μ M) or cycloheximide (1 μ M) with either IL-1 β (50 ng/ml) or PDGF-BB (20 ng/ml) for 24 h. Binding assays were performed in triplicate with concentrations of [3 H]BK ranging from 0.2 to 6 nM and incubated at 4 $^{\circ}$ C for 4 h. Data are expressed as means $\pm$ S.E.M. from at least three individual experiments (n). * $P < 0.01$, compared with control untreated cells; † $P < 0.01$, compared with control cells treated with IL-1 β or PDGF.

Treatment	K_D (nM)	B_{max} (fmol/mg of protein)
Control		
Untreated	0.91 $\pm$ 0.17	30 $\pm$ 4 ($n = 7$)
IL-1 β	1.03 $\pm$ 0.07	119 $\pm$ 5* ($n = 3$)
PDGF	1.22 $\pm$ 0.09	52 $\pm$ 7* ($n = 5$)
PD98059		
Untreated	1.16 $\pm$ 0.34	30 $\pm$ 13 ($n = 4$)
IL-1 β	1.02 $\pm$ 0.22	35 $\pm$ 5† ($n = 5$)
PDGF	1.27 $\pm$ 0.17	19 $\pm$ 12† ($n = 3$)
Cycloheximide		
Untreated	1.12 $\pm$ 0.23	49 $\pm$ 7 ($n = 3$)
IL-1 β	1.13 $\pm$ 0.21	56 $\pm$ 10† ($n = 3$)

Table 3 Relative potencies of drugs for [3 H]BK binding to canine TSMCs preincubated with IL-1 β

Cultured TSMCs were preincubated in the presence of IL-1 β (50 ng/ml) for 24 h. [3 H]BK (5 nM) binding was determined as described in the Materials and methods section. Values are means $\pm$ S.E.M. from three separate experiments determined in triplicate.

Drug	IC ₅₀ (nM)	K_i (nM)
Agonist		
des-Arg ⁹ -BK	> 100 000	> 100 000
BK	7.5 $\pm$ 0.5	3.0 $\pm$ 0.3
Antagonist		
[Leu ⁸ , des-Arg ⁹]BK	> 100 000	> 100 000
Hoe 140	6.9 $\pm$ 0.6	2.9 $\pm$ 0.2

cells treated with these two reagents for 24 h, using [3 H]BK as a radioligand. As shown in Table 2, when TSMCs were cultured with IL-1 β and PDGF-BB for 24 h, the B_{max} values significantly increased from 30 $\pm$ 4 (untreated cells) to 119 $\pm$ 5 (IL-1 β -treated cells) and 52 $\pm$ 7 (PDGF-BB-treated cells) fmol/mg of protein. These increases in [3 H]BK binding sites evoked by IL-1 β and PDGF-BB were significantly reversed by concurrent incubation with PD98059. Furthermore, the site of action of IL-1 β might be associated with BK receptor synthesis, since cycloheximide reversed this effect (Table 2). The K_D values were not changed significantly compared with those of the control cells.

To examine further which BK receptor subtype was increased in the cells treated with IL-1 β for 24 h, the selective B₁ agonist and antagonist, des-Arg⁹-BK and [Leu⁸, des-Arg⁹]BK (where des means 'deletion of amino acid from the respective peptide'), and selective B₂ agonist and antagonist, BK and Hoe 140, were used to competitively inhibit [3 H]BK binding to TSMCs. All tested drugs inhibited [3 H]BK binding with the same affinities as those of the control (Table 3). [3 H]BK binding to TSMCs treated with IL-1 β was inhibited by the B₂ receptor-selective agonist and antagonist, BK and Hoe 140, but not affected by the B₁ receptor-selective agonist and antagonist, des-Arg⁹-BK and [Leu⁸, des-Arg⁹]BK. Although the B₁ receptor-selective reagents inhibited [3 H]BK binding by $\approx$ 10% at the concentration of 100 μ M, this concentration was greater than their K_i values. These results

Table 4 Requirement of Ras and Raf for IL-1 β -induced up-regulation of BK receptors in TSMCs

Cells were transfected without (control) or with plasmids encoding pZIP-NeoSV, H-Ras-15A, pCGN or Raf-N4, as indicated in the Materials and methods section, and then incubated with IL-1 β (50 ng/ml) for 24 h. Binding assays were performed in triplicate with concentrations of [3 H]BK ranging from 0.2 to 6 nM and incubated at 4 °C for 4 h. Data are expressed as means $\pm$ S.E.M. from three individual experiments. * P < 0.01, compared with control untreated cells.

Treatment	K_D (nM)	B_{max} (fmol/mg of protein)
Control	0.92 $\pm$ 0.21	149 $\pm$ 25
PZIP-NeoSV	0.81 $\pm$ 0.19	135 $\pm$ 31
H-Ras-15A	0.83 $\pm$ 0.29	46 $\pm$ 16*
pCGN	0.74 $\pm$ 0.36	126 $\pm$ 27
Raf-N4	0.81 $\pm$ 0.25	65 $\pm$ 11*

suggest that the increase in receptor density is primarily due to the B $_2$ receptor.

Up-regulation of BK receptors through the activation of Ras and Raf-1

Several lines of evidence have suggested that Ras plays an important role in a variety of cell functions mediated through sequential activation of Raf-1, MEK1/2 and MAPK [37–39]. To elucidate whether the activation of Ras/Raf is required for up-regulation of BK receptor density induced by IL-1 β , TSMCs were transfected with or without a dominant negative Ras (H-Ras-15A) or Raf (Raf-N4) and then treated with IL-1 β . As shown in Table 4, IL-1 β induced an increase in BK receptor density in TSMCs, which was inhibited by transfection with H-Ras-15A or Raf-N4. There was no significant change when TSMCs were transfected with empty vectors pZIP-NeoSV and pCGN, respectively. These results demonstrated that IL-1 β caused an up-regulation of BK receptors through activation of the Ras/Raf/MEK/MAPK pathway in TSMCs.

DISCUSSION

Several lines of evidence suggest that IL-1 β plays an important role in the regulation of cellular signalling cascades associated with airway inflammation in asthma [5,6,8]. In addition, IL-1 β may also contribute to enhanced airway constrictor responsiveness *in vivo* [22,23]. However, the mechanism(s) of IL-1 β involved in airway hyper-responsiveness are not completely understood. The purpose of this study was to determine the mechanisms by which IL-1 β enhanced cellular responses to BK stimulation in canine cultured TSMCs. We demonstrate that (i) BK is a more effective stimulator of Ins(X)*P* accumulation and Ca $^{2+}$ mobilization in canine TSMCs exposed to IL-1 β , although treatment of TSMCs with IL-1 β did not enhance the carbachol-, ET-1- and 5-HT-induced Ins(X)*P* response; (ii) the action of IL-1 β might be attributed to an increase in BK receptor synthesis, which is, at least in part, mediated through the activation of p42/p44 MAPK, since cycloheximide and PD98059 reversed these effects; and (iii) the selective increase in the responsiveness of TSMCs to BK upon treatment of cells with IL-1 β and PDGF-BB appears to result largely from an increase in the number of BK receptors. The mechanism underlying the enhancing effect of IL-1 β most probably shares a common signalling pathway with PDGF-BB.

Because IL-1 β was found to stimulate cell proliferation [11,12], the subsequent increase in the thickness of the bronchial wall

apparently contributed to the development of airway hyper-responsiveness and the increase in airway resistance in response to bronchoconstricting stimuli [22,23]. In addition, elevated levels of IL-1 β have been found in bronchoalveolar lavage fluid in symptomatic asthmatic subjects [5,6]. Because of a defect in the respiratory epithelium lining [10], which is observed in asthma, it is likely that IL-1 β may have relatively easy access to the TSMCs. The fact that IL-1 β also increased [3 H]thymidine incorporation in TSMCs, e.g. that IL-1 β increased smooth-muscle mass, may have additional important clinical implications [11,12]. Our results obtained with IL-1 β are consistent with previous studies showing that the accumulation of Ins(X)*P* and Ca $^{2+}$ mobilization induced by BK were enhanced by tumour necrosis factor- α treatment in human cultured TSMCs [40] and human epidermal carcinoma cells [41]. Although the mechanisms of enhancement remain to be determined, there is evidence that one of the mechanisms might be involved in the augmentation of transmembrane signalling. In this study, the potentiating effect of IL-1 β was dependent on the incubation time and its concentrations. Short-term treatment with IL-1 β is not sufficient to cause augmentation of Ins(X)*P* accumulation and Ca $^{2+}$ mobilization induced by BK. However, long-term treatment (24 h) with IL-1 β apparently enhances the BK-induced responses in these cells. Indeed, the enhancing effects induced by IL-1 β were abolished by concurrent exposure to cycloheximide. It can be concluded that the enhancing effects of IL-1 β on BK receptor-linked PtdIns/phospholipase C activation in canine TSMCs require the induction of protein synthesis. Moreover, the concentration–effect curves of BK-induced Ins(X)*P* accumulation and Ca $^{2+}$ mobilization were shifted to the left and the maximal responses were significantly enhanced in TSMCs treated with IL-1 β as compared with the control cells. The EC $_{50}$ values of BK-induced Ins(X)*P* accumulation and Ca $^{2+}$ mobilization in TSMCs treated with IL-1 β were close to those of control cells. These results suggest that treatment with IL-1 β did not change the affinity of BK receptors.

Although mitogenic signals from the activation of specific tyrosine kinase-coupled growth factors have been well characterized, the mechanism by which the cytokines activate the MAPK cascade effectors is not known. PD98059 is a synthetic and highly specific inhibitor of MEK1/2 activation [42]. Because activation of components in the MAPK cascade originates from stimulation of cells by growth factors, it has been proposed that transmission of the signal along this pathway is required for the regulation of cellular responses. In current study, pretreatment with PD98059 attenuated IL-1 β -induced expression of BK receptors in TSMCs, revealing that stimulation of MEK1/2 is required for IL-1 β -induced responses in these cells. However, PD98059 partially inhibited the enhancement of IL-1 β on Ins(X)*P* accumulation induced by BK, implying that an alternative pathway activated by IL-1 β may be implicated in this effect.

Furthermore, the enhancement of IL-1 β on cellular responses might involve an up-regulation of cell-surface receptor density or a change in the affinity. Several studies reported that IL-1 β increases the expression of cell-surface BK receptors [28,35,36] and thereby enhances cell signalling in response to various stimuli. In the present study, we found that treatment with IL-1 β and PDGF-BB increased total [3 H]BK binding sites in canine TSMCs. We speculate that IL-1 β and PDGF-BB particularly increase the BK receptor number at the gene level, associated with protein synthesis, since TSMCs require to be exposed to IL-1 β for several hours before any enhancing effect appears and is inhibited by cycloheximide. Although the concentration of cycloheximide used in this study cannot exclude the activation of a stress-response pathway, it would demonstrate that inhibition

of protein synthesis may be a candidate mechanism. Furthermore, the B₂ receptor-selective reagents competed with [³H]BK binding in a concentration-dependent manner. BK has a high affinity for B₂ and a low affinity for B₁ receptors [43]. In contrast, des-Arg⁹-BK has a high affinity for B₁, but a low affinity for B₂ receptors [43]. In this study, the B₁ receptor-selective agonist, des-Arg⁹-BK, and antagonist, [Leu⁸, des-Arg⁹]BK, triggered no significant displacement at concentrations up to 100 μ M, thus excluding the presence of B₁ receptors in canine TSMCs. In contrast, the competitive inhibition of [³H]BK binding by the B₂ receptor-selective antagonist Hoe 140 showed a single population of BK binding sites with a high affinity (2.9 ± 0.2 nM) for B₂ receptors in canine TSMCs treated with IL-1 β , confirming its high affinity for B₂ receptors described in other tissues [44]. Thus the increase in BK receptors induced by IL-1 β is primarily of B₂ receptors in TSMCs. This hypothesis is supported by the evidence that treatment with IL-1 β did not affect carbachol-, ET-1- and 5-HT-induced Ins(X)P accumulation. It is therefore conceivable that IL-1 β specifically induces an increase in the number of BK receptors, which in turn potentiates BK-induced Ins(X)P accumulation and Ca²⁺ mobilization in canine cultured TSMCs.

It has been well established that growth factors activate phosphorylation of protein kinases, including tyrosine kinases, Ras, Raf-1, MEK and MAPK [37–39]. The activation of protein kinases generally plays an important role in cell growth and gene expression [37–39]. Several studies have suggested that MAPK could be a convergence point for growth signals originating from tyrosine kinase receptors, G-protein-coupled receptors and cytokines [37–39]. In TSMCs, it has been reported that MAPK activation plays an important role for PDGF- and ET-1-induced cell proliferation [18,45]. Several lines of evidence have suggested that Ras is activated by various stimuli for growth and differentiation [46,47] and that the activated Ras evoked the phosphorylation cascade of protein kinases including Raf-1, MEK1/2 and MAPK [37–39]. It has been demonstrated that, in TSMCs, PDGF-BB-induced cell proliferation may be mediated through the activation of Ras [38]. Thus it remains to be determined whether Ras plays a key role in the up-regulation of BK receptors induced by IL-1 β in TSMCs. In this study, to elucidate whether Ras is involved in an increase in BK receptor number induced by IL-1 β , TSMCs were transfected with H-Ras-15A, which interacts preferentially with guanine nucleotide-exchange factors and inhibits Ras functions [48,49]. We found that IL-1 β -induced up-regulation of BK receptors was significantly suppressed by transfection with the dominant negative mutant of Ras (H-Ras-15A) in TSMCs, as reported previously in other cell types [15,49]. Several studies have also shown that activated Ras binds and activates Raf-1, resulting in the activation of MEK and MAPK [47,50]. Consistent with these reports, we demonstrated in TSMCs that IL-1 β -induced up-regulation of BK receptors was significantly inhibited by transfection with the dominant negative mutant of Raf-1 (Raf-N4), suggesting that Raf-1 plays a key role in IL-1 β -induced up-regulation of BK receptors in TSMCs.

In conclusion, these results demonstrate that treatment with IL-1 β induces an increase in BK receptor expression and reinforces BK-induced Ins(X)P accumulation and Ca²⁺ mobilization in canine cultured TSMCs. These enhancements by IL-1 β and PDGF-BB might be due to an up-regulation of BK B₂ receptor density (B_{max}), since [³H]BK binding to TSMCs was inhibited by the B₂ receptor-selective agonist and antagonist, BK and Hoe 140, but not by the B₁-selective reagents. Furthermore, overexpression of dominant negative mutants, H-Ras-15A and Raf-N4, significantly suppressed the up-regulation of BK receptors induced by IL-1 β , indicating that Ras and Raf may be

required for activation of these kinases. Taken together, these studies suggest that IL-1 β exerts a number of biological actions in the airways, at least in part through the activation of the Ras/Raf/MEK/MAPK pathway in TSMCs. Our results present an example of signal-transduction-mediated modulation of receptor expression by IL-1 β , associated with potentiation of agonist responses in canine cultured TSMCs.

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