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Regulation of kinin receptors in airway epithelial cells by inflammatory cytokines and dexamethasone

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Abstract

The two kinin receptors, B_1 and B_2, are upregulated in inflammation and may play a role in diseases such as asthma. In pulmonary A549 cells, TNF-α or interleukin-1β dramatically increased bradykinin B_1 and B_2 receptor mRNA expression and this response was prevented by dexamethasone. In primary human bronchial epithelial cells, bradykinin B_1 receptor mRNA expression showed a similar trend, whereas bradykinin B_2 receptor showed almost constitutive expression. Radioligand-binding studies revealed significant increases in bradykinin B_2 receptor protein expression following both interleukin-1β and TNF-α treatment of A549 cells; however, no evidence was found for bradykinin B_1 receptor. Functionally, the bradykinin B_2 receptor ligand, bradykinin, but not the B_1 ligand, des-Arg^{10}-kallidin, produced a marked increase in prostaglandin E_2 release when administered following interleukin-1β treatment. Arachidonic acid release in response to bradykinin was markedly enhanced by prior incubation with interleukin-1β and this was prevented by the prior addition of dexamethasone.

Keywords: Bradykinin; Des-Arg^{10}-kallidin; Bradykinin B_1 receptor; Bradykinin B_2 receptor; Cyclooxygenase; Epithelial cell; Dexamethasone

1. Introduction

Bradykinin and the related kinin, lys-bradykinin (kallidin), are formed following inflammatory insult or tissue injury (Bhoola et al., 1992; Proud and Kaplan, 1988). These two peptides exert their effects via the G-protein coupled bradykinin B_2 receptor and are rapidly redigested by carboxypeptidases to produce des-Arg^9-BK and des-Arg^{10}-kallidin (Lys-des-Arg^9-bradykinin), which act via the closely related bradykinin B_1 receptor (Marceau et al., 1998). The bradykinin B_2 receptor is constitutively expressed by most cells and is thought to play a role in nonpathological conditions as well as in acute phase inflammatory and pain responses. By contrast, the bradykinin B_1 receptor is highly inducible and may be expressed in chronic inflammation. This possibility was highlighted in a model of allergic inflammation, where bradykinin B_1 receptor expression in the lungs was increased following allergen exposure, and bronchial hyperresponsiveness was inhibited by a bradykinin B_1 receptor antagonist (Huang et al., 1999). Furthermore, bradykinin B_1 receptor may play a role in the elaboration of TNF-α and interleukin-1β from macrophage cells (Tiffany and Burch, 1989). In the airways, kinins mediate multiple effects, including bronchoconstriction, particularly, in asthmatics (Fuller et al., 1987; Polosa and Holgate, 1990), vascular dilatation and plasma exudation (Fox et al., 1996; Hogan et al., 1997; Ichinose and Barnes, 1990). Thus, a role for kinins in inflammatory diseases, such as asthma, is likely (Marceau et al., 1998; Proud, 1998). This idea is supported by the finding that elevated levels of kinins, and kininogen precursors, occur in the bronchoalveolar fluid of asthmatics (Christiansen et al., 1987). Furthermore, kinin levels were significantly increased by allergen challenge in asthmatics and pro-inflammatory cytokines increased receptor expression in lung fibroblast and smooth muscle cells (Christiansen et al., 1992; Haddad et al., 2000; Schmidlin et al., 1998).

Airway epithelial cells not only form a protective lining in the airways, but may also contribute to inflammation by the production of lipid mediators and pro-inflammatory

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cytokines (Chung and Barnes, 1999). Whilst bradykinin is known to stimulate the production of arachidonic acid and prostaglandin E_2 in pulmonary A549 cells (Saunders et al., 1999; Tokumoto et al., 1994), little is known about the regulation of the bradykinin receptors. As A549 cells provide a model, in terms of prostanoid production, for pulmonary epithelial cells (Mitchell et al., 1994), we have used these cells and primary bronchial epithelial cells to further investigate the expression and function of the bradykinin receptors.

2. Materials and methods

2.1. Cell culture

A549 cells (ATCC, Rockville, MD) were grown to confluence as previously described (Newton et al., 1998). Cells were incubated overnight in serum-free medium prior to changing to fresh medium containing cytokines and/or drugs as indicated.

Primary bronchial epithelial cells, obtained from bronchial brushings from normal individuals undergoing fiberoptic bronchoscopy and attending as out-patients at the Royal Brompton Hospital, were prepared according to the previously described methods (Donnelly and Barnes, 2001; Kelsen et al., 1992). The Royal Brompton Hospital Ethics Committee approved the protocol and all subjects gave informed consent. Resultant cells were cultured as described previously (Donnelly and Barnes, 2001). Once confluent, cells were incubated in supplement-free media for 24 h prior to changing to supplement-free media containing cytokines and/or drugs as indicated.

Interleukin-1β (R&D Systems Europe, Abbingdon, Oxon), TNF-α (R&D Systems) bradykinin (Sigma, Pool, Dorset), des-Arg^{10}-kallidin (Sigma), des-Arg^{9}-[Leu^{8}]-bradykinin (Sigma), HOE 140 (Sigma), dexamethasone (Sigma), PD098059 (2′-amino-3′ methoxyflavone) (Alexis, Bingham, UK), and SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) (Alexis) were all dissolved in either media or in dimethly sulphoxide and diluted at least 1:1000 in media prior to use.

2.2. Semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA was extracted from primary epithelial cells using QIAGEN RNeasy kits (QIAGEN, UK). Reverse transcription reactions, PCR conditions and primers for GAPDH were as previously described (Newton et al., 1997a). PCR primers for human bradykinin B_1 and B_2 receptors were (5′ to 3′): bradykinin B_1 receptor, TGT GAA CGC CTT CAT TTT CTG C (sense) and AAC AAA TTG GCC TTG ATG ACC C (antisense); bradykinin B_2 receptor, TCA CAT CCC ACT CTG AGT CC (sense) and GTC CAA GGG GTC CAT CTA GA (antisense). Product sizes were 519 and 318 bp, respectively, and identity was confirmed by sequencing. Cycling parameters were: 94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min. In each case, the exponential phase of the reaction, where starting material is proportional to product formation, was determined as previously described (Newton et al., 1997a). Reaction products were analysed by agarose gel electrophoresis and gel images subject to densitometric analysis using GelWorks 1D Version 4.01 (NonLinear Dynamics) (UVP, Cambridge, UK).

2.3. Northern blot analysis

Total RNA was prepared as previously described (Haddad et al., 2000; Newton et al., 1998), prior to purification of Poly $(A)^+$ RNA using the PolyATract mRNA isolation system (Promega, Southampton, UK). The resultant mRNA was size-fractionated on 1% agarose/formaldehyde gels and subjected to northern hybridisation using probes derived from the cloned bradykinin B_1 and B_2 receptors and GAPDH PCR products (above) and conditions previously described (Haddad et al., 2000; Newton et al., 1998).

2.4. Radioligand binding

Cells were treated as indicated and membrane preparations were carried out as previously described (Haddad et al., 2000). Binding studies were also as described previously using the ligands [^{3}H]bradykinin (NEN, Hounslow, UK) or [^{3}H]des-Arg^{10}-kallidin (NEN) and nonspecific binding was determined in the presence of 5 μM unlabelled ligand (Haddad et al., 2000). All determinations were carried out in triplicate.

2.5. Radioimmunoassay (RIA) determination of prostaglandin E_2 release

Radioimmunoassay (RIA) for prostaglandin E_2 in the supernatants was according to the manufacturer's instructions (Sigma). Determination of combined cyclooxygenase and prostaglandin E synthase activity was performed as previously described (Mitchell et al., 1994; Neri et al., 1998). Cells were washed before incubation at 37 °C in media containing 30 μM arachidonic acid (Sigma). After 30 min, the media was removed and assayed for prostaglandin E_2. The level of prostaglandin E_2 was taken as an index of combined cyclooxygenase plus prostaglandin E synthase activity.

2.6. [^{3}H] arachidonic acid release

Assessment of arachidonic acid release was carried out as previously described (Newton et al., 2000). Confluent cells in 24-well plates were incubated overnight in 0.5 ml serum-free medium containing 0.125 μCi [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]arachidonic acid (Amersham Pharmacia). Cells were washed two times in fresh media prior to treatment with

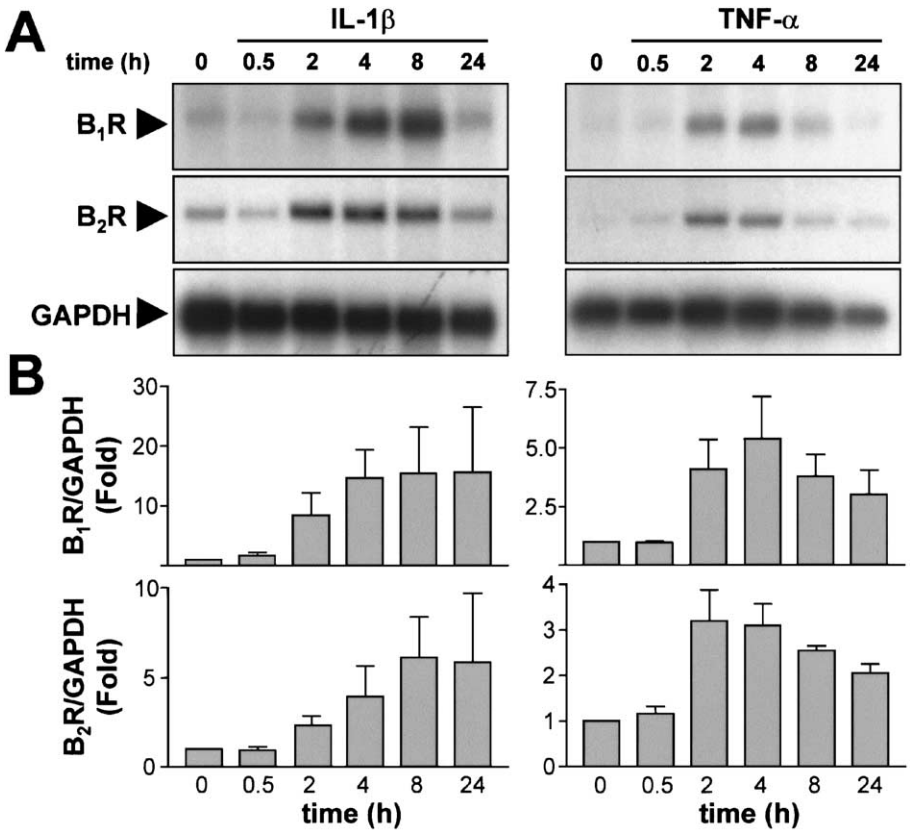

Fig. 1. Regulation of bradykinin B_1 and B_2 receptor mRNA by interleukin-1β and TNF-α. A549 cells were treated with interleukin-1β (IL-1β) (1 ng/ml) or TNF-α (10 ng/ml) for the times indicated. Cells were harvested and RNA was prepared for northern analysis of bradykinin B_1 and B_2 receptor mRNAs (B_1R and B_2R, respectively) and GAPDH. (A) Autoradiographs representative of 3 (interleukin-1β) or 5 (TNF-α) experiments are shown. (B) After densitometric analysis, data were normalised to GAPDH, expressed as fold induction, and plotted as means ± S.E.M.

dexamethasone (1 μM) for 1 h prior to interleukin-1β (1 ng/ml) as indicated. After a further 6 h, the media was changed to fresh media containing 2 mg/ml fatty acid-free bovine serum albumin (Sigma). Cells were then treated with dexamethasone, bradykinin or des-Arg^{10}-kallidin as indicated. After 30 min, the supernatants were harvested for liquid scintillation counting and the cells harvested in 1% sodium dodecyl sulphate for liquid scintillation counting. [^{3}H]arachidonic acid, and its metabolites, released during the 30 min were expressed as percentage of the total incorporated.

3. Results

3.1. A549 cells express bradykinin B_1 and B_2 receptor mRNA in response to interleukin-1β, TNF-α and cycloheximide

Northern blot analysis revealed low levels of expression of both bradykinin B_1 and B_2 receptors in unstimulated A549 cells (Fig. 1). Treatment with either interleukin-1β or TNF-α dramatically increased mRNA expression of both receptors. In each case induction of bradykinin B_1 receptor mRNA was greater than for bradykinin B_2 receptor owing to the typically higher basal level of bradykinin B_2 receptor mRNA. Addition of the protein synthesis inhibitor, cyclo-

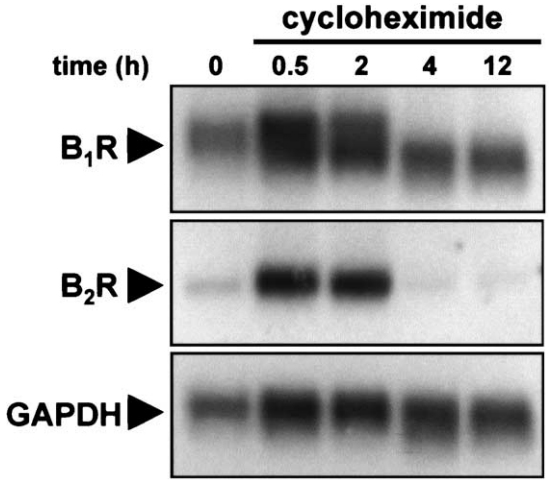

Fig. 2. Induction of bradykinin B_1 and B_2 receptor mRNA by cycloheximide. A549 cells were treated with cycloheximide (10 μg/ml) for the times indicated. Cells were harvested and RNA was prepared for northern analysis of bradykinin B_1 and B_2 receptor mRNAs (B_1R and B_2R, respectively) and GAPDH. Autoradiographs representative of two such experiments are shown.

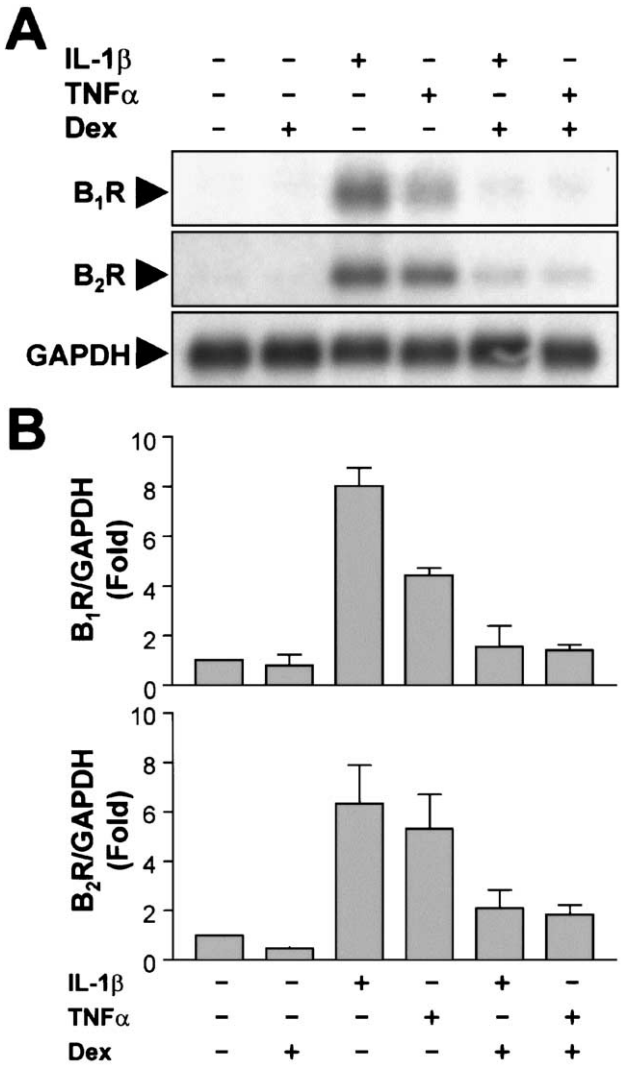

Fig. 3. Effect of dexamethasone on the induction of bradykinin B_1 and B_2 receptor mRNA. A549 cells were pretreated with dexamethasone (1 μM) (Dex) for 1 h and stimulated with either interleukin-1β (IL-1β) (1 ng/ml) or TNF-α (10 ng/ml) as indicated. Cells were harvested after 4 h and RNA was prepared for northern analysis of bradykinin B_1 and B_2 receptor mRNAs (B_1R and B_2R, respectively) and GAPDH. (A) Autoradiographs representative of three such experiments are shown. (B) After densitometric analysis, data were normalised to GAPDH, expressed as fold induction, and plotted as means ± S.E.M.

heximide, resulted in a very rapid induction of both bradykinin B_1 and B_2 receptor mRNA expression (Fig. 2).

3.2. Dexamethasone represses bradykinin B_1 and B_2 receptor expression

Pretreatment of A549 cells with dexamethasone almost totally prevented the ability of both interleukin-1β and TNF-α to induce mRNA expression of bradykinin B_1 and B_2 receptors (Fig. 3). These data suggest that prevention of kinin receptor expression may also represent a mechanism of action for anti-inflammatory glucocorticoids.

3.3. Human bronchial epithelial cells express kinin receptor mRNA

To examine these responses in cells that are physiologically relevant to airway inflammation, primary human bronchial epithelial cells were stimulated with interleukin-1β and TNF-α and the effect of dexamethasone was examined (Fig. 4). In these cells both kinin receptor mRNAs were found to be expressed and in each case, interleukin-1β and TNF-α tended to result in increased expression. Furthermore, this trend was, to some extent, reduced by pretreatment with dexamethasone. However, these results were variable as many of the primary epithelial cultures exhibited very high basal expression of both bradykinin B_1 and B_2

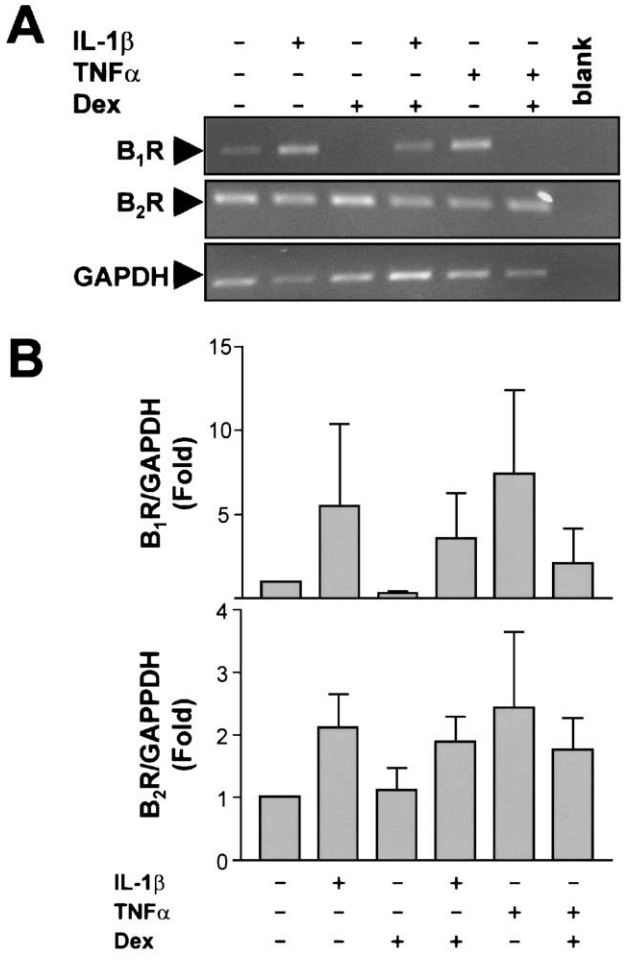

Fig. 4. Effect of interluekin-1β, TNF-α and dexamethasone on kinin receptor expression in primary bronchial epithelial cells. Primary human bronchial epithelial cells were stimulated with either TNF-α (10 ng/ml) or interleukin-1β (1 ng/ml) with or without a 30-min pretreatment with dexamethasone (1 μM) (Dex) as indicated. Cells were harvested after 4 h and RNA was prepared for semiquantitative RT-PCR analysis of bradykinin B_1 and B_2 receptor mRNAs (B_1R and B_2R, respectively) and GAPDH. (A) Representative ethidium-bromide-stained gels are shown. (B) After densitometric analysis, data ($n = 3–8$) were normalised to GAPDH, expressed as fold induction, and plotted as means ± S.E.M.

receptors and, in these instances, expression was not further inducible (data not shown).

3.4. Determination of receptor expression by radioligand binding

Using [^{3}H]bradykinin for ligand binding, as previously described (Haddad et al., 2000), there was a low basal level of specific binding (B_{max} 21 fmol/mg protein) that was dramatically enhanced by treatment with TNF-α or interleukin-1β for up to 24 h (B_{max} 82.2 and 95.5 fmol/mg protein, respectively) (Fig. 5). As has been reported in previous studies, single-site binding analysis revealed that the K_d value did not change with stimulation, indicating that there was no change in receptor affinity (data not shown). To confirm that this binding was specific for the bradykinin B_2 receptor, cells were treated with TNF-α and harvested after 24 h. [^{3}H]bradykinin binding was analysed in the presence of various concentrations of either HOE 140, a selective bradykinin B_2 receptor antagonist, or des-Arg^9-[Leu^8]-bradykinin, a selective bradykinin B_1 receptor antagonist (Fig. 5C). Specific binding was almost totally prevented by HOE 140, but not with des-Arg^9-[Leu^8]-bradykinin, indicating that this effect was specific to bradykinin B_2 receptor. By contrast, analysis of binding using [^{3}H]des-Arg^{10}-kallidin, as previously described (Haddad et al., 2000), revealed only very low levels of specific binding (data not shown). Even following interleukin-1β stimulation, specific binding represented no more than 10–20% of total binding, suggesting that bradykinin B_1 receptor protein was not present.

To examine the effect of dexamethasone on bradykinin B_2 receptor expression, cells ($n=2$ in duplicate) were treated as in Fig. 3 with interleukin-1β (1 ng/ml) in the presence or absence of dexamethasone (1 μM). After 24 h, cells were harvested and radioligand binding using [^{3}H]bradykinin as ligand performed as in Fig. 5. Untreated cells showed specific binding of 22.3 ± 2.1 fmol/mg protein and this was increased to 87.6 ± 5.5 fmol/mg protein by interleukin-1β, which is consistent with Fig. 5B. Dexamethasone reduced the interleukin-1β-induced binding to 28.5 ± 3.9 fmol/mg protein, indicating the potent repressive effect of glucocorticoids.

3.5. Bradykinin induces prostaglandin E_2 release from interleukin-1β-primed cells

As both bradykinin B_1 and B_2 receptor-dependent responses may lead to enhanced release of prostaglandins (Galizzi et al., 1994; Saunders et al., 1999), we tested the ability of both bradykinin and des-Arg^{10}-kallidin to stimulate release of prostaglandin E_2. However, in A549 cells, the ability to produce prostaglandin E_2 is dependent on the presence of cyclooxygenase-2, and prostaglandin E synthase, which are both induced by pro-inflammatory stimuli (Jakobsson et al., 1999; Mitchell et al., 1994; Newton et al., 1997a; Thoren and Jakobsson, 2000). Thus, following stimulation with interleukin-1β, A549 cells produce prostaglandin E_2 in a cumulative time-dependent manner (Fig. 6A). This increase in prostaglandin E_2 release lags behind the induction of combined cyclooxygenase and prostaglandin E synthase activity, which is not present in unstimulated cells and is revealed by the addition of exogenous arachidonic acid to bypass the endogenous phospholipase A_2 step (Fig 6B). The fact that in the presence of arachidonic acid for 30 min, interleukin-1β-stimulated cells produce large quantities of prostaglandin E_2 indicates that once cyclooxygenase and prostaglandin E synthase activities are induced, then phospholipase A_2 activity or arachidonate availability becomes limiting (compare Fig. 6A and B). Likewise, cells that have been stimulated with interleukin-1β alone give rise to low or undetectable levels of PGE_2 release during a 30-min period prior to harvesting (Fig. 6C).

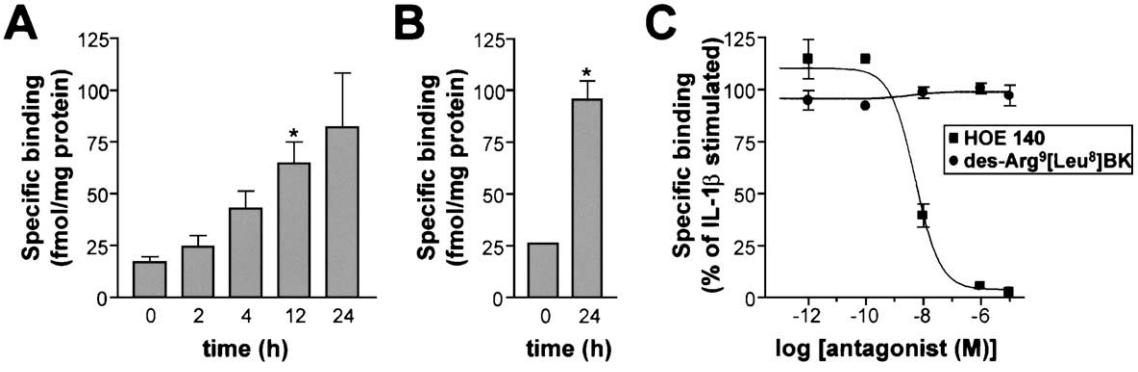

Fig. 5. Interleukin-1β and TNF-α increase bradykinin B_2 receptor numbers. (A) A549 cells were treated with TNF-α (10 ng/ml) for the times indicated. Cells were harvested and crude membranes were prepared for radioligand binding using [^{3}H]bradykinin. Specific binding (B_{max}) data from four separate determinations, each carried out in triplicate, are plotted as means ± S.E.M. Significance was tested by ANOVA with Dunn's post-test. * $P<0.05$. (B) Cells were stimulated, or not, with interleukin-1β (1 ng/ml) and harvested after 24 h for radioligand binding analysis. Data from four separate determinations, each performed in triplicate, are plotted as means ± S.E.M. Significance was tested by Mann–Whitney U test. * $P<0.05$. (C) Membranes prepared from cells treated with TNF-α (10 ng/ml) for 24 h were subject to radioligand binding analysis in the presence of various concentrations of des-Arg^9-[Leu^8]-bradykinin (desArg^9[Leu^8]BK) or HOE 140 as indicated. Data from two separate determinations, performed in triplicate, are shown as means ± S.E.M.

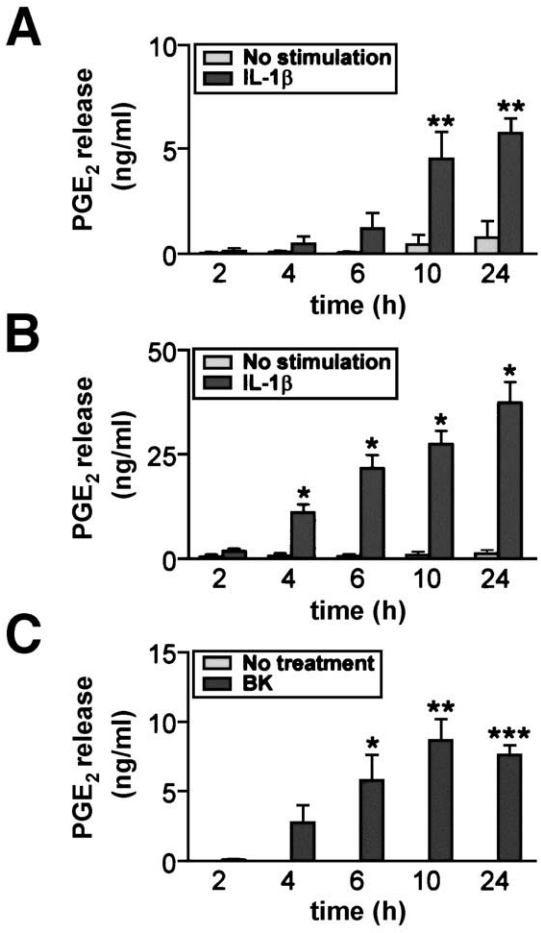

Fig. 6. Induction of prostaglandin E_2 release by bradykinin in combination with interleukin-1β. A549 cells were either not stimulated or treated with interleukin-1β (1 ng/ml) for the times indicated for the following treatments and assays. (A) Supernatants were harvested and released, prostaglandin E_2 was determined by RIA. (B) Supernatants were removed and cells were washed prior to the addition of 30 μM arachidonic acid for 30 min and subsequent determination of released prostaglandin E_2. (C) Cells that had been prestimulated with interleukin-1β for the times indicated were either not treated or treated with bradykinin (1 μM) (BK) for 30 min prior to prostaglandin E_2 determination. All data ($n=6$) are plotted as means ± S.E.M. Significance in (A) and (B) was tested by Mann–Whitney U test and by Wilcoxon signed rank test in (C). * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

This again suggests that the induction of phospholipase A_2 activity by interleukin-1β is poor and is consistent with our previous findings (Newton et al., 2000). Addition of bradykinin for 30 min to cells that had previously stimulated with interleukin-1β resulted in a profound induction of prostaglandin E_2 synthesis (Fig. 6C). This effect was not observed with des-Arg^{10}-kallidin (data not shown). Similarly, the simultaneous addition of interleukin-1β (1 ng/ml), bradykinin (1 μM) or both together results in 1.04 ± 0.40, 0 ± 0 and 5.66 ± 1.37 ng/ml prostaglandin E_2, respectively, after 6 h ($n=5$) and 6.92 ± 0.67, 0 ± 0 and 11.46 ± 0.68 ng/ml after 24 h ($n=8$) treatment. These data again demonstrate the combinatorial, synergistic, effect of interleukin-1β and bradykinin on release of prostaglandin E_2. As there was no obvious effect of simultaneous addition of bradykinin on cyclooxygenase/prostaglandin E synthase activity (data not shown), these data suggest that the effect of bradykinin lies primarily in inducing release of arachidonic acid in this system.

3.6. Release of prostaglandin E_2 by bradykinin is inhibited by HOE 140

In cells that had been stimulated with interleukin-1β, bradykinin evoked a dose-dependent release of prostaglandin E_2 (EC_{50} 0.0012 μM) (Fig. 7A). In addition, the bradykinin B_1 receptor agonist, des-Arg^{10}-kallidin, also produced a rise in prostaglandin E_2 release at concentrations over 1 μM. To examine the specificity of this response, cells were stimulated with interleukin-1β for 24 h before treating with 100 μM des-Arg^{10}-kallidin in the presence or absence of selective antagonists (Fig. 7B). Prostaglandin E_2 release was totally prevented by the bradykinin B_2 receptor antagonist, HOE 140, but was unaffected by the bradykinin B_1 receptor antagonist, des-Arg^9-[Leu^8]-bradykinin, suggesting that the response was a nonspecific effect of des-Arg^{10}-kallidin acting via the bradykinin B_2 receptor rather than a bona fides bradykinin B_1 receptor-dependent response (Fig. 7B). By contrast, the response to bradykinin was unaffected by bradykinin B_1 receptor antagonists, but was blocked by HOE 140, confirming that this effect was specific to bradykinin B_2 receptor (Fig. 7C).

To examine whether this response to bradykinin was indeed mediated by cyclooxygenase-2, cells were treated for 24 h with interleukin-1β prior to treating with 1 μM bradykinin in the presence of various doses of either indomethacin, a combined cyclooxygenase-1/cyclooxygenase-2 inhibitor, or NS-398, a selective cyclooxygenase-2 inhibitor. These experiments ($n=2$) produced EC_{50} values of 0.08 and 0.011 μM, respectively, which taken with published values for inhibition of cyclooxygenase-1, 0.013 μM (indomethacin) and 12 μM (NS-398), and cyclooxygenase-2, 0.044 μM (indomethacin) and 0.0095 μM (NS-398) (Kawai et al., 1998), strongly suggest that bradykinin-induced prostaglandin E_2 occurs via cyclooxygenase-2 and not cyclooxygenase-1.

3.7. Bradykinin induces release of arachidonate and repression by dexamethasone

Release of arachidonic acid is a prerequisite for prostanoid production and may be induced by both bradykinin B_1 and B_2 receptors (Haddad et al., 2000; Tropea et al., 1993). Cells were, therefore, loaded overnight with [^{3}H]arachidonic acid prior to stimulating with interleukin-1β as indicated (Fig. 8). After 6 h, bradykinin, but not des-Arg^{10}-kallidin, appeared to be able to induce release of [^{3}H]arachidonate from both unstimulated and interleukin-1β-stimulated cells. However, in unstimulated cells, this effect was not significant, whilst in interleukin-1β-treated cells, the substantially

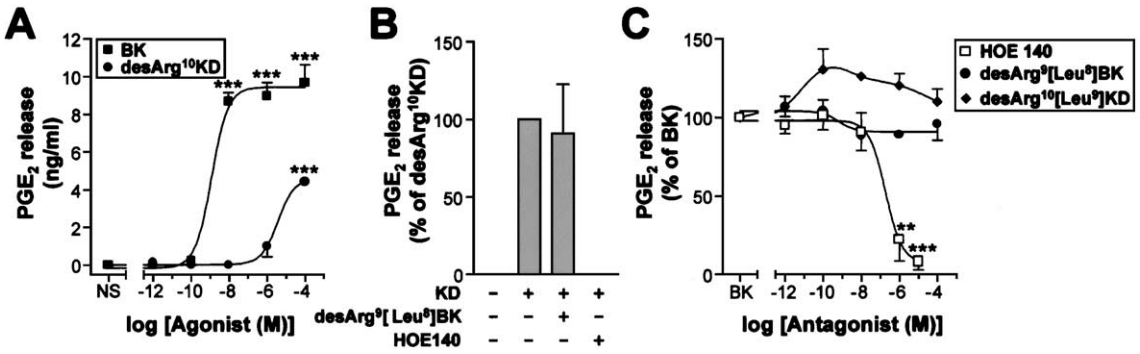

Fig. 7. The role of bradykinin B_1 and B_2 receptors in the prostaglandin E_2 release induced by des-Arg^{10}-kallidin and bradykinin. (A) Cells were incubated with interleukin-1β (1 ng/ml) for 24 h prior to washing and treatment with various doses of either bradykinin (BK) or des-Arg^{10}-kalladin (desArg^{10}KD). After 30 min, supernatants were collected for prostaglandin E_2 determination. (B) Cells were stimulated with interleukin-1β as in (A) prior to treatment with des-Arg^{10}-kallidin (100 μM) (desArg^{10}KD) in the presence or absence of either des-Arg^{9}-[Leu^{8}]-bradykinin (100 μM) (desArg^{9}[Leu^{8}]BK) or HOE 140 (100 μM). Supernatants were collected after 30 min for prostaglandin E_2 determination. (C) After stimulation with interleukin-1β as in (A), cells were treated with bradykinin (0.01 μM) (BK) in the presence or absence of various concentrations of HOE 140, des-Arg^{9}-[Leu^{8}]-bradykinin (desArg^{9}[Leu^{8}]BK) or des-Arg^{10}-[Leu^{9}]-kallidin (desArg^{10}[Leu^{9}]KD). After 30 min, supernatants were collected for prostaglandin E_2 determination. Data in (A) (BK, $n=4$; desArg^{10}KD, $n=5$), (B) ($n=4$) and (C) (HOE 140, $n=4$; desArg^{9}[leu^{8}]BK, $n=3$; desArg^{10}[Leu^{9}]KD, $n=3$) are plotted as means ± S.E.M. Significance was tested by ANOVA using a Bonferroni post-test. ** $P<0.01$, *** $P<0.001$.

greater levels of release are consistent with the increased expression of bradykinin B_2 receptor as indicated by radioligand binding (Fig. 5). Addition of dexamethasone 1 h prior to the interleukin-1β ($t=-1$) totally prevented the increased response to interleukin-1β plus bradykinin (Fig. 8B). By contrast, when dexamethasone was added just prior to the bradykinin, i.e. at the end of the 6 h interleukin-1β treatment ($t=6$), there was no obvious effect on the ability of bradykinin to release [^{3}H]arachidonate. These data suggest that in the presence of bradykinin B_2 receptor (induced by interleukin-1β), dexamethasone has no direct effect on the processes that lead to arachidonic acid release by bradykinin, but rather exerts an effect on gene expression by preventing basal and IL-1β-induced expression of bradykinin B_2 receptor.

3.8. Role of the ERK and p38 mitogen activated kinase pathways

A549 cells were treated as in Fig. 7C except that various concentrations of the selective mitogen activated protein kinase (MAPK)/extracellular regulated kinase (ERK) kinase 1 (MEK1) inhibitor, PD098059, or the p38 MAPK inhibitor, SB203580, were used in place of antagonists. PD098059 revealed a concentration-dependent inhibition of bradykinin-

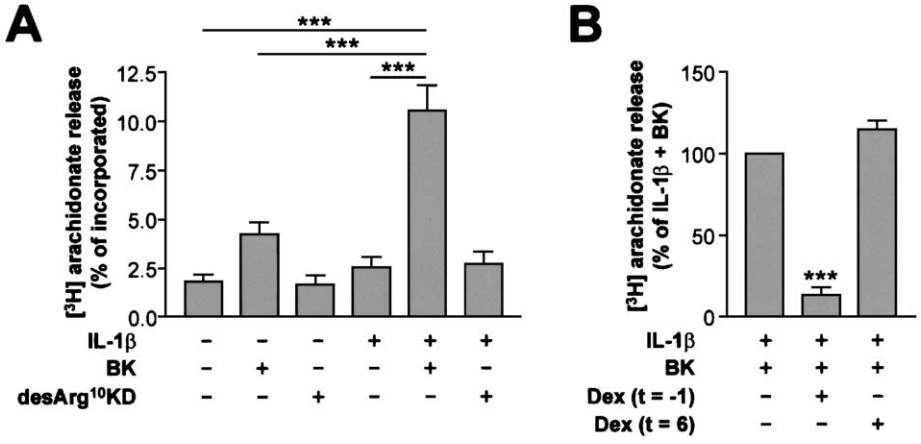

Fig. 8. A combination of interleukin-1β plus bradykinin results in release of arachidonic acid. (A) After loading with [^{3}H]arachidonic acid, cells were either not stimulated or stimulated with interleukin-1β (1 ng/ml) for 6 h. Supernatants were then removed and new media added prior to not treating, or treating cells with bradykinin (1 μM) (BK) or des-Arg^{10}-kallidin (1 μM) (desArg^{10}KD), as indicated. After 30 min, supernatants and cells were harvested for liquid scintillation counting. Data ($n=8-10$) are expressed as arachidonate release as a percentage of the total incorporated and are plotted as means ± S.E.M. Significance was tested by ANOVA with a Dunn's post-test. (B) Cells were treated with interleukin-1β for 6 h as in (A). Dexamethasone (1 μM) (Dex) was added either 1 h before the interleukin-1β ($t=-1$) or immediately prior to BK ($t=6$) as indicated. After 30 min, supernatants and cells were harvested as above. Data ($n=7$) are expressed as arachidonate release as a percentage of interleukin-1β + BK treated and are plotted as means ± S.E.M. Significance was tested by Mann–Whitney U test. *** $P<0.001$.

induced prostaglandin E_2 release with an EC_{50} value of between 30 and 100 μM (data not shown). However, the published IC_{50} value for MEK1 inhibition by PD098059 is 2–7 μM and, in A549 cells, this inhibitor totally prevented interleukin-1β-induced ERK phosphorylation at 10 μM, indicating that the MEK1-ERK pathway is unlikely to be involved in the bradykinin-induced release of prostaglandin E_2 (Alessi et al., 1995; Newton et al., 2000). Similarly, SB203580 showed less than 50% inhibition of bradykinin-induced PGE_2 release at 10 μM (data not shown). As the reported IC_{50} for inhibition of p38 MAPK is 0.6 μM (Cuenda et al., 1995) and, in intact cells, including A549 cells (Newton et al., 2000), functional responses are observed at this concentration, we conclude that the p38 MAPK does not play a role in this response.

4. Discussion

In the present study, we have used A549 pulmonary cells to demonstrate upregulation of bradykinin B_1 receptor and, to a lesser extent, bradykinin B_2 receptor mRNA expression by the pro-inflammatory cytokines, interleukin-1β and TNF-α. The kinetics of mRNA induction in these cells are similar to those of other primary response genes, such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 and granulocyte/macrophage colony-stimulating factor, and are consistent with a role for acute phase transcriptional activators (Fig. 1) (Bergmann et al., 2000; Newton et al., 1996, 1997a, 1998). In particular, the transcription factor nuclear factor-κB (NF-κB) has been implicated in the regulation of bradykinin B_1 receptor mRNA expression and has been previously shown to be rapidly and potently upregulated by both interleukin-1β and TNF-α in these cells (Newton et al., 1996; Ni et al., 1998). In addition, the protein synthesis inhibitor, cycloheximide, was able to very rapidly induce mRNA expression of both kinin receptors (Fig. 2). This effect has previously been reported in fibroblasts and may be explained by the existence of a labile repressor protein that normally acts to maintain basal mRNA expression in unstimulated cells (Haddad et al., 2000; Phagoo et al., 2000; Zhou et al., 1998). In the case of bradykinin B_1 receptor mRNA expression, cycloheximide has been previously shown to stabilise the mRNA (Zhou et al., 1998). However, this mRNA induction may also occur transcriptionally via the activation of transcription factors, such as NF-κB, which is negatively regulated by the labile inhibitor, IκBα, and has shown to be activated by cycloheximide in A549 cells (Newton et al., 1997b). In common with other pro-inflammatory genes, such as cyclooxygenase-2 or granulocyte/macrophage colony-stimulating factor (Bergmann et al., 2000; Newton et al., 1997a, 1998), the induction of bradykinin B_1 and B_2 receptor mRNA expression in response to interleukin-1β and TNF-α, in these cells, was blocked by the anti-inflammatory glucocorticoid, dexamethasone (Fig. 3).

In primary human epithelial cells, mRNA expression of both bradykinin B_1 and B_2 receptors was also observed (Fig. 4). In the case of bradykinin B_2 receptor, this was essentially constitutive, whereas bradykinin B_1 receptor appeared to be more highly inducible by both interleukin-1β and TNF-α. In both cases, dexamethasone also showed some repressive effect. Overall, these effects were found to be highly variable, presumably due to heterogeneity in human populations as well as the possibly of differing conditions of the donor cells. Unfortunately, the relative paucity of available primary airway epithelial cells precluded any further functional analysis. However, taken with the A549 data, these studies provide evidence for (i) mRNA expression of both kinin receptors in inflammatory conditions, (ii) targeting of this pathway at the level of receptor expression by anti-inflammatory glucocorticoids, and (iii) the occurrence of these events in primary airway epithelial cells.

Radioligand-binding studies in the A549 cells indicated the presence and upregulation of bradykinin B_2 receptor, but not bradykinin B_1 receptor, by TNF-α and interleukin-1β (Fig. 5). These data were functionally corroborated by the finding that bradykinin, but not des-Arg^{10}-kallidin, was able to induce arachidonic acid and prostaglandin E_2 release from interleukin-1β-treated cells (see Figs. 6 and 8). Taken together, these data suggest, in contrast to our previous findings in fibroblast cells (Haddad et al., 2000), that in A549 cells upregulation of the bradykinin B_1 receptor mRNA does not lead to protein expression. Since the conclusion of our study, Phagoo et al. (2001) have reported an upregulation of bradykinin B_1 receptor following interleukin-1β treatment of A549 cells (Phagoo et al., 2001). Whilst these findings are clearly inconsistent with the data presented here, we would like to emphasise that our data, in contrast to Phagoo et al. (2001), are supported by relevant functional measurements, which support the conclusion that B_1R is either not expressed or expressed at a level that is too low for functional significance. Furthermore, we also note that the specific [^{3}H]des-Arg^{10}-kallidin binding data of Phagoo are expressed as a percentage of control and no actual data on receptor numbers is given (Phagoo et al., 2001). Consequently, we are unable to comment on the absolute receptor numbers in the two studies.

In these studies, we demonstrate that the induction of bradykinin B_2 receptor by interleukin-1β allows cells to respond to exogenous bradykinin by producing large quantities of prostaglandin E_2 (Fig. 6). Conversion of exogenous arachidonic acid to prostaglandin E_2 revealed that this increased release is not due to an effect of bradykinin on the expression/activity of cyclooxygenase or the downstream prostaglandin E synthase, but is due to enhanced arachidonic acid release, presumably via activation of one or more phospholipase A_2 enzymes. This effect is demonstrated in Fig. 8. In A549 cells, the release of prostaglandin E_2 following interleukin-1β stimulation occurs via cyclooxygenase-2 and not via cyclooxygenase-1, which shows low mRNA and protein expression (Mitchell et al., 1994; New-

ton et al., 1997a). Consistent with this, we find that induction of prostaglandin E_2 release by bradykinin treatment of interleukin-1β-stimulated cells is potently inhibited by the cyclooxygenase-2 selective inhibitor, NS-398. This finding is also consistent with previous studies and indicates that interleukin-1β-treatment results in the upregulation of both bradykinin B_2 receptor and cyclooxygenase-2, which act in combination to synergistically induce prostaglandin E_2 release in response to bradykinin (Saunders et al., 1999). Thus, in inflammatory conditions, upregulation of cyclooxygenase-2 and prostaglandin E synthase requires the coordinate upregulation of bradykinin B_2 receptors to allow strong activation of phospholipase A_2 and concomitant high-level prostaglandin E_2 production.

We have previously implicated the ERK and p38 MAPKs in the induction of prostaglandin E_2 from A549 cells by interleukin-1β by virtue of either direct activation of phospholipase A_2 or in the coupling of phospholipase A_2 to cyclooxygenase-2 (Newton et al., 2000). However, the data presented here do not support a role for either MEK1 or p38 MAPKs in the release of prostaglandin E_2 by bradykinin. Furthermore, we demonstrate that in addition to repression at the mRNA level (Fig. 3), the upregulation of bradykinin B_2 receptors by interleukin-1β is prevented at the receptor level by pretreatment with the glucocorticoid, dexamethasone. This accounts for the ability of dexamethasone to prevent stimulation of arachidonic acid release by BK (Fig. 8). Thus, dexamethasone represses the ability of these cells to produce prostaglandin E_2 by preventing bradykinin-dependent activation of phospholipase A_2 as well as the previously documented profound repression of cyclooxygenase-2, which occurs via transcriptional and post-transcriptional mechanisms (Newton et al., 1998).

In summary, we have documented the gene expression of bradykinin B_1 and B_2 receptors in primary human airway epithelial cells and demonstrated upregulation by inflammatory stimuli. In addition, we found that the bradykinin B_2 receptor is functionally coupled to arachidonic acid and prostaglandin E_2 release and show that this is prevented by dexamethasone. We, therefore, conclude: (i) that bradykinin B_2 receptor plays a role in airway inflammation by enhancing the production of prostaglandin E_2 from epithelial cells, and (ii) that this pathway is targeted at multiple levels by glucocorticoids such as dexamethasone.

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