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# Regulation of bradykinin receptor gene expression in human lung fibroblasts

Stephen B. Phagoo [1], Mohammed Yaqoob, Esteban Herrera-Martinez, Peter McIntyre, Caroline Jones, Gillian M. Burgess [*, 2]

*Novartis Institute for Medical Sciences, 5 Gower Place, London WC1E 6BN, UK*

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**Abstract**

In WI-38 human fibroblasts, interleukin-1β and tumour necrosis factor-α (TNF-α) increased bradykinin $B_1$ receptor mRNA, which peaked between 2 and 4 h, remaining elevated for 20 h. Binding of the bradykinin $B_1$ receptor selective ligand [$^3$H]des-Arg$^{10}$-kallidin, also increased, peaking at 4 h and remaining elevated for 20 h. The $B_{max}$ value for [$^3$H]des-Arg$^{10}$-kallidin rose from 280 ± 102 fmol/mg ($n = 3$) to 701 ± 147 fmol/mg ($n = 3$), but the $K_D$ value remained unaltered (control, 1.04 ± 0.33 nM ($n = 3$); interleukin-1β, 0.88 ± 0.41 nM ($n = 3$)). The interleukin-1β-induced [$^3$H]des-Arg$^{10}$-kallidin binding sites were functional receptors, as bradykinin $B_1$ receptor agonist-induced responses increased in treated cells. Bradykinin $B_2$ receptor mRNA and [$^3$H]bradykinin binding were upregulated by interleukin-1β, but not TNF-α. The effect of interleukin-1β on bradykinin $B_2$ receptors was smaller than for bradykinin $B_1$ receptors. Cycloheximide prevented interleukin-1β-mediated increases in $B_1$ and $B_2$ binding, but not mRNA suggesting that de novo synthesis of a transcriptional activator was unnecessary. 

*Keywords:* Bradykinin $B_1$ receptor; Bradykinin $B_2$ receptor; Regulation; [$^3$H]des-Arg$^{10}$-kallidin; [$^3$H]Bradykinin; WI-38 cell; Cytokine

## 1. Introduction

Bradykinin and kallidin (Lys-bradykinin) are proteolytically released by circulating kallikreins during tissue injury and inflammation. They play a key role in a number of pathophysiological processes including inflammatory pain (Proud and Kaplan, 1988; Hall, 1992; Dray and Perkins, 1993; Dray, 1997; Marceau et al., 1998).

Two types of bradykinin receptor subtypes, bradykinin $B_1$ receptors (Menke et al., 1994) and bradykinin $B_2$ receptors (Hess et al., 1992), have been cloned and pharmacologically defined (Regoli and Barabe, 1980). Both subtypes belong to the G-protein-coupled superfamily of receptors. Bradykinin $B_1$ receptors are generally absent under non-pathological conditions and are rapidly synthesised de novo following tissue damage or inflammation (Marceau, 1995; Marceau et al., 1998). In contrast, bradykinin $B_2$ receptors are expressed constitutively in a wide range of tissues (Hall, 1992). The carboxypeptidase metabolites of bradykinin and kallidin, des-Arg$^9$-bradykinin and des-Arg$^{10}$-kallidin, respectively, are selective agonists at the bradykinin $B_1$ receptor and have little or no activity at bradykinin $B_2$ receptors.

A feature of the bradykinin $B_1$ receptor, which has attracted much interest, is its upregulation under conditions of tissue injury, a phenomenon initially studied in rabbit vascular tissues (Marceau, 1995). There is strong evidence that bradykinin $B_1$ receptors are involved in animal models of persistent inflammatory hyperalgesia (Farmer et al., 1991; Davis and Perkins, 1994; Perkins and Kelly, 1994; Perkins et al., 1995) and other pathological conditions (Marceau et al., 1998), and studies such as these have demonstrated that the inflammatory cytokine interleukin-1β is pivotal in the processes that result in bradykinin $B_1$ receptor upregulation. Despite the involvement of the

* Corresponding author. Discovery Biology, Pfizer, Ramsgate Road, Sandwich, Kent CT13 9NJ, England, UK. Tel.: +44-1304-646057; fax: +44-1304-655619.

*E-mail address:* gillian_burgess@sandwich.pfizer.com (G.M. Burgess).

[1] Current address: Childrens Hospital Los Angeles Research Institute, 4650 Sunset Boulevard, CA 90027, USA.

[2] Current address: Pfizer, Sandwich, Kent CT13 9NJ, UK.

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closely related cytokine tumour necrosis factor-α (TNF-α) in inflammation, it has been reported that this cytokine does not play a role in bradykinin-$B_1$-receptor-mediated inflammatory hyperalgesia (Davis and Perkins, 1994; Perkins and Kelly, 1994). In order to study the relative abilities of these two cytokines to upregulate bradykinin $B_1$ receptor mRNA and protein in the same cell type, we have examined cytokine-induced regulation of bradykinin $B_1$ and $B_2$ receptor gene expression in WI-38 fibroblast cells. Regulation was assessed at both the transcriptional and protein levels following exposure of the cells to both interleukin-1β and TNF-α. For comparison, the expression of bradykinin $B_2$ receptors was also examined.

## 2. Materials and methods

### 2.1. Culture of human foetal lung fibroblasts

WI-38 fibroblasts were grown as described previously (Phagoo et al., 1996). For whole-cell binding experiments, the cells were plated at a density of 200,000 cells/well in six-well (3 cm) plates and allowed to grow to confluence (2–3 days). For $^{45}Ca^{2+}$ efflux studies, fibroblasts were plated at a density of 60,000 cells/well in 96-well plates. For mRNA analysis, a single confluent flask (175 $cm^2$) was used for each condition. After reaching confluency, WI-38 cells were exposed to interleukin-1β (100 IU/ml) or TNF-α (350 RU/ml) in the existing media for various times from 0.5 to 20 h as described in the figure legends.

### 2.2. WI-38 membrane preparation

WI-38 cells were harvested as described previously in the presence of a cocktail of peptidase inhibitors (Phagoo et al., 1996). The membranes were frozen on dry ice, then stored at −70°C until use. The protein concentration was determined by the method of Bradford (1976) using a BioRad kit (Biorad). Immediately prior to use, frozen membrane aliquots were thawed in binding buffer and mixed to give a homogenous membrane suspension.

### 2.3. Radioligand binding assays

Membrane binding assays: [$^3$H]des-Arg$^{10}$-kallidin saturation binding assays were performed as described previously (Phagoo et al., 1996). Briefly, WI-38 membranes (usually 30–60 μg protein/ml) were incubated for 1 h in the presence of a range of concentrations of [$^3$H]des-Arg$^{10}$-kallidin from 0.03 to 6 nM in binding buffer containing 10 mM *N*-Tris [Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) buffer pH 7.4, 0.14 g/l bacitracin, 1 mM 1,10 phenanthroline and 1 g/l bovine serum albumin at 4°C. Non-specific binding was defined as the amount of labelled ligand bound in the presence of 1 μM des-Arg$^{10}$-kallidin. All incubations were terminated by rapid filtration through GF/B glass fibre filtermats that had been pre-soaked for at least 2 h in 6 g/l polyethylenimine, using a Brandel 48-cell harvester. The tubes and filters were washed five times with 1 ml aliquots of 50 mM Tris[hydroxymethyl]amino-methane (Tris) buffer (pH 7.4, 4°C) before counting for radioactivity. Whole-cell binding assays: Cells in six-well plates were incubated in binding buffer (as above) with the addition of 300 mM glucosamine in the presence of 1 nM [$^3$H]des-Arg$^{10}$-kallidin. After incubation for 1 h at 4°C, the assay buffer was removed and the cells washed with three 3-ml aliquots of ice-cold 50 mM Tris containing 300 mM sucrose (pH 7.4). The cells were then lysed with 0.05% sodium dodecyl sulphate (SDS). A small sample was removed from each well for protein estimation by the method of Bradford before counting for radioactivity. [$^3$H]Bradykinin binding experiments were performed as described above, with substitution of [$^3$H]bradykinin for [$^3$H]des-Arg$^{10}$-kallidin and non-specific binding measured in the presence of 1 μM unlabelled bradykinin. All assays were carried out in triplicate and the variation between wells was $< 7\%$. Specific binding was expressed in femtomoles per milligram of protein and processed using ORIGIN (Microcal Software, USA).

### 2.4. $^{45}Ca^{2+}$ efflux

WI-38 cells were loaded with $^{45}Ca^{2+}$ (100 μCi/ml) in 50 μl of growth medium and incubated at 37°C for $> 18$ h. The cells were washed 10 times over a period of 20 min with Hanks Balanced Salt Solution containing 10 mM HEPES (37°C, pH 7.4) (wash buffer) using an eight-row cell washer. After this period, the rate of $^{45}Ca^{2+}$ efflux was stable. Cells were exposed to wash buffer (control) or des-Arg$^{10}$-kallidin (100 nM) made up in wash buffer at 37°C for 4 min before removing a 50 μl sample from each. For antagonist studies, the last two washes were replaced with antagonist (4 min total) followed by a 4-min exposure to antagonist in the presence of agonist prior to sampling. The $^{45}Ca^{2+}$ remaining in the cells at the end of the experiment was assessed by lysing the cells with 0.2% SDS. Microscint-40 (200 μl) was added to the samples, which were counted for radioactivity. The increase in $^{45}Ca^{2+}$ efflux induced by the agonist has been expressed as disintegrations per minute above the background.

### 2.5. mRNA analysis by Northern blotting

Total RNA was extracted from the cells using the acid guanidinium–thiocyanate–phenol–chloroform technique described by Chomczynski and Sacchi (1987). The purity of the total RNA was calculated by determining the absorbance at 260 and 280 nm. Samples (5 μl) of total RNA (1 mg/ml) were mixed with 10 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), 4 mM sodium acetate, 0.5

mM ethylenediaminetetraacetate (EDTA), 2 M formaldehyde, and 50% formamide, pH 8.0. The samples were then heated at 65°C for 15 min then mixed with 2 μl sterile sample buffer (comprising 5% glycerol, 1 mM EDTA, 0.25% bromophenol blue, and 1 ml of 1 mg/ml ethidium bromide). The samples were loaded onto agarose gels (1%), which were run for 4–5 h at 50 V and photographed under UV light. The gels were washed for 20 min in 50 mM NaOH and rinsed in water equilibrated with 20× sodium citrate sodium chloride (SSC). The gels were then blotted overnight onto positively charged nylon membranes (Gene screen PLUS, DuPont) that had been pre-equilibrated with 20× SSC. The following day the membranes were rinsed in 10× SSC, dried at room temperature and baked at 80°C for 2 h.

Human bradykinin $B_1$ receptor $^{32}$P-labelled cDNA probe was prepared by digesting a plasmid containing Human bradykinin $B_1$ receptor cDNA (Jones et al., 1999) with *Eco*RI and *Xba* and the resulting 1.2-kb fragment spanning the open frame was purified from a 1% agarose gel using Jetsorb (Genomed). DNA was labelled with a Megaprime kit (Amersham). Briefly, 50 ng of the purified bradykinin $B_1$ receptor DNA fragment was mixed with 19 μl of $H_2O$, 5 μl of random octamer primers, boiled for 5 min then chilled. Deoxyribonucleotides (4 μl 2.5 mM dCTP, dGTP and dTTP) were added to the DNA in the

Table 1
Saturation binding data for [$^3$H]des-Arg$^{10}$-kallidin in control and interleukin-1β treated WI-38 cells
Cells were treated with interleukin-1β, (100 IU/ml) for 2 h prior to harvesting and preparing membranes. The maximum receptor density ($B_{max}$) and equilibrium dissociation constant ($K_D$) were estimated by non-linear curve fitting using ORIGIN. Data are means ± sem of three experiments performed in triplicate.

| | $K_D$ (nM) | $B_{max}$ (fmol/mg) |
|---|---|---|
| Control | 1.04 ± 0.33 | 280 ± 102 |
| Interleukin-1β | 0.88 ± 0.41 | 701 ± 147** |

** $P < 0.01$, paired Student's *t*-test compared to control cells.

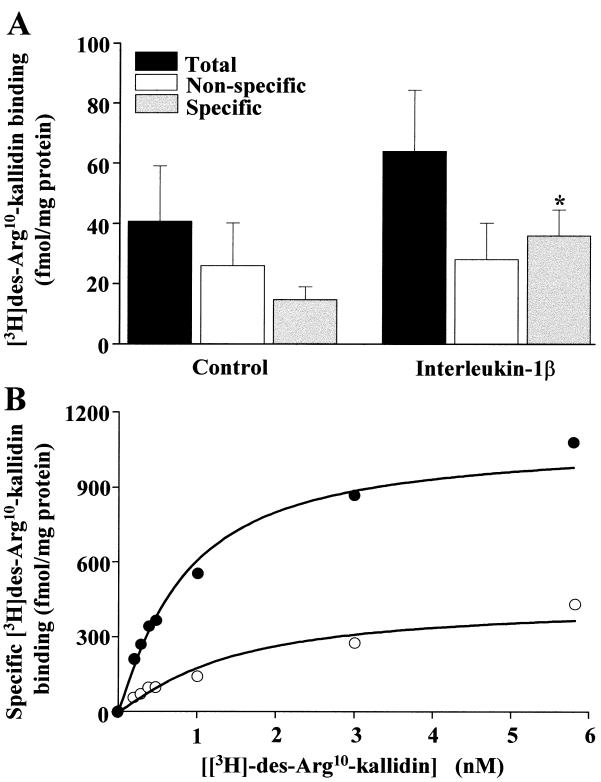


Fig. 1. Effect of interleukin-1β on specific [$^3$H]des-Arg$^{10}$-kallidin binding in WI-38 cells. (A) Intact cells were treated for 2 h with interleukin-1β (100 IU/ml) then incubated with [$^3$H]des-Arg$^{10}$-kallidin (1 nM). The binding data in whole cells represent the means ± sem of four independent experiments, each performed in triplicate. * $P < 0.05$, Student's *t*-test compared to untreated cells. (B) Saturation isotherms for [$^3$H]des-Arg$^{10}$-kallidin in WI-38 membranes prepared from untreated cells (○) or cells pre-treated with interleukin-1β (100 IU/ml) for 2 h (●). The results shown are from one of three experiments, which gave similar results. Each point was performed in triplicate and the $B_{max}$ and $K_D$ values obtained from the analysis of all three experiments are shown in Table 1.

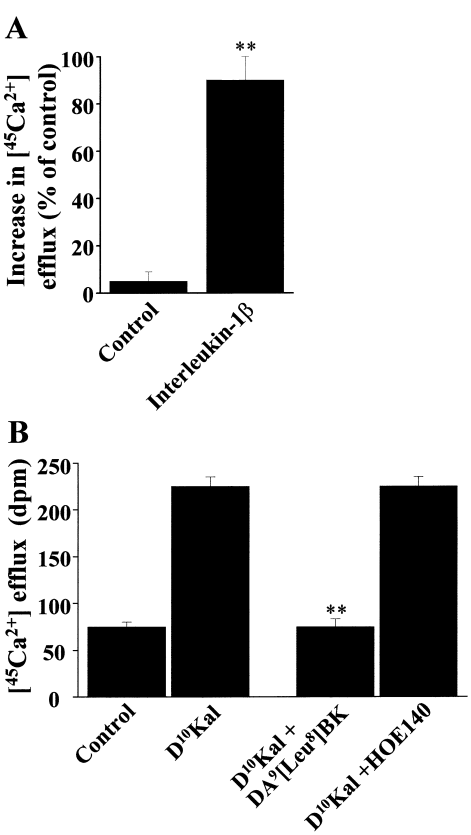


Fig. 2. Effect of interleukin-1β on des-Arg$^{10}$-kallidin induced efflux of $^{45}Ca^{2+}$ from WI-38 cells. (A) Cells were treated for 2 h with interleukin-1β (100 IU/ml) then stimulated with des-Arg$^{10}$-kallidin (D$^{10}$Kal, 100 nM). ** $P < 0.01$ Student's *t*-test, compared to control cells. (B) Effect of the bradykinin $B_1$ receptor antagonist, des-Arg$^9$[Leu$^8$]-bradykinin (DA$^9$[Leu$^8$]BK; 30 μM) and the bradykinin $B_2$ receptor antagonist, HOE140 (10 μM) on des-Arg$^{10}$-kallidin induced efflux of [$^{45}Ca^{2+}$]. Values represent the means ± sem ($n = 4$–6). ** $P < 0.01$ Studen'ts *t*-test compared to the response to 100 nM des-Arg$^{10}$-kallidin in the absence of antagonist.

presence of [$^{32}$P]dATP and the Klenow fragment of DNA polymerase 1. This mixture was incubated at 37°C for 30 min, then stopped by the addition of 50 μl stop buffer (comprising: Tris–HCl 50 mM; EDTA, 10 mM; NaCl, 100 mM; SDS, 0.1%, pH 8.6). The $^{32}$P-labelled DNA probe was then separated from the free nucleotides and the [$^{32}$P]dATP on a Sephadex G50 column. The human bradykinin $B_2$ receptor mRNA was probed using a $^{32}$P-labelled DNA probe prepared as described above from a plasmid containing the 1.3-kb full-length fragment from rat bradykinin $B_2$ receptor cDNA (McIntyre et al., 1993). Northern blots were rehybridised with a control 0.75 kb β-actin probe to confirm the loading and integrity of the samples.

The membranes were pre-hybridised with hybridisation buffer (comprising: formamide, 50%; dextran sulphate, 10%; SDS, 1%; boiled herring sperm DNA, 100 mg; Denhardt's reagent, 5%; (Ficoll, 0.02%; polyvinylpyrrolidone, 0.02%; bovine serum albumin, 0.02%); NaCl, 0.75 M and tri-sodium citrate, 0.075 M) that had been heated at 65°C for 15 min. The nylon membranes were pre-hybridised with this solution for 1 h at 42°C. The $^{32}$P-labelled DNA probes ($4–5 \times 10^5$ dis/min/ml) were heated in 1 ml of the hybridisation solution at 100°C for 5 min then transferred to the blot and left to hybridise for 16 h at 42°C. The following day, the blots were washed under low stringency conditions ($0.2 \times$ SSC for 15 min at 55°C) then exposed to X-ray film (Biomax, 5 h). The bands on the

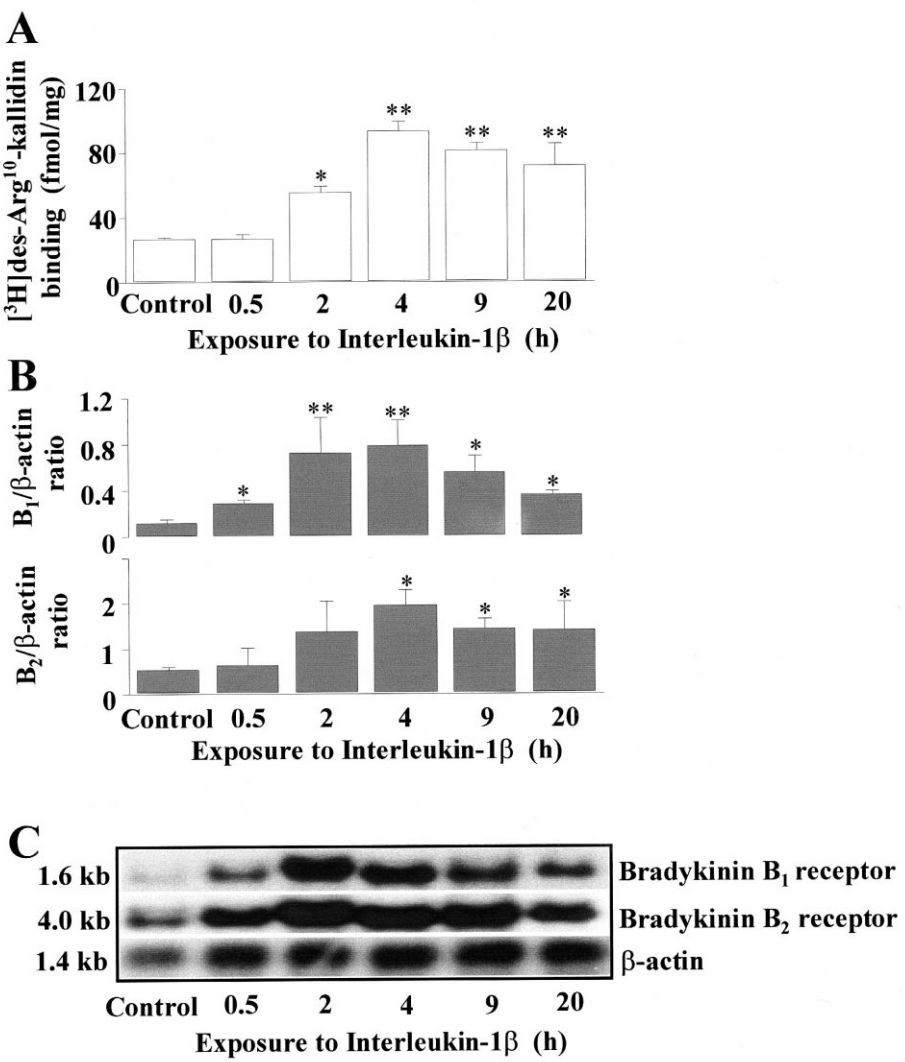


Fig. 3. Time-course of the effect of interleukin-1β on $B_1$ and $B_2$ mRNA levels and specific [$^3$H]des-Arg$^{10}$-kallidin in WI-38 cells. The cells were treated with interleukin-1β (100 IU/ml) for various times as indicated. (A) Specific [$^3$H]des-Arg$^{10}$-kallidin binding to intact WI-38 cells. The data represent the means ± sem of three independent experiments. (B) Quantitative Northern analysis of phosphorimager counts for $B_1$ (top panel) and $B_2$ mRNA signals (bottom panel) calculated as the ratio of β-actin mRNA signal. The data represent the means ± sem of three independent experiments. (C) A representative Northern blot analysis for $B_1$ mRNA (top panel), $B_2$ mRNA (middle panel) and β-actin mRNA (bottom panel). $^{*}P < 0.05$, $^{**}P < 0.01$ Student's *t*-test, comparison of specific binding or phosphorimager ratios in interleukin-1β-treated cells and control cells.

resulting autoradiographs were quantified by phosphorimager (BIO-RAD GS 250 Molecular Imager).

### 2.6. Materials

[$^3$H]Bradykinin (specific activity 65 Ci/mmol) and $^{45}Ca^{2+}$ (specific activity 5–50 mCi/mg $Ca^{2+}$) were obtained from Amersham International (Amersham UK, Bucks). [$^3$H]des-Arg$^{10}$-kallidin (specific activity 107–110 Ci/mmol) was provided by DuPont NEN (Hertfordshire, UK). Bradykinin and other peptides were obtained from Peninsula Laboratories Europe, (Merseyside, UK). WI-38 fibroblasts were obtained from ICN Biomedicals, (Oxon, UK) and used between passage number 19 and 25. Culture reagents and Hanks Buffered Salt Solution were obtained from Gibco (Scotland). Human recombinant TNF-α (35,200 Reference Units (RU)/μg) was obtained from Promega and human recombinant interleukin-1β (100,000 International Units (IU)/μg) from the National Institute for Biological Standards and Control, Hertfordshire, UK. All other reagents were obtained from Sigma (Poole, UK).

## 3. Results

### 3.1. Upregulation of bradykinin $B_1$ and $B_2$ receptors in WI-38 cells by interleukin-1β

It has previously been shown that WI-38 cells express bradykinin $B_1$ and $B_2$ receptor mRNA (Webb et al., 1994) and protein (Phagoo et al., 1996). Under normal culture conditions, the bradykinin $B_1$ and $B_2$ receptor subtypes are present at a ratio of approximately 1:3 (Phagoo et al., 1996). When the WI-38 cells were treated with interleukin-1β (100 IU/ml) for 2 h (Fig. 1A), there was a two- to threefold increase in specific binding of the bradykinin $B_1$ receptor selective ligand, [$^3$H]des-Arg$^{10}$-kallidin (1 nM), from a control level of $15 \pm 4$ fmol/mg of protein ($n = 3$) to a stimulated level of $36 \pm 9$ fmol/mg of protein ($n = 3$). In order to determine whether the increase in [$^3$H]des-Arg$^{10}$-kallidin binding following interleukin-1β treatment was due to an increase in the receptor density ($B_{max}$) or a shift in the equilibrium dissociation constant ($K_D$) for the radioligand, saturation isotherms were measured in membranes prepared from control cells and from cells that had been pre-treated with interleukin-1β (100 IU/ml) for 2 h. Interleukin-1β did not change the $K_D$ value for [$^3$H]des-Arg$^{10}$-kallidin, but the $B_{max}$ value increased by a factor of 2.5-fold ($P < 0.01$, paired Student's *t*-test) in the treated cells (Table 1; Fig. 1B). Under basal conditions, the bradykinin $B_1$ receptor agonist des-Arg$^{10}$-kallidin (100 nM) caused a small increase in [$^{45}Ca^{2+}$] release from WI-38 cells (see Fig. 2). This increase in the specific binding of [$^3$H]des-Arg$^{10}$-kallidin was mirrored by an increase in the size of the [$^{45}Ca^{2+}$] response to des-Arg$^{10}$-kallidin (Fig. 2A). The response to des-Arg$^{10}$-kallidin in interleukin-1β-treated cells was completely inhibited by the bradykinin $B_1$ receptor antagonist des-Arg$^9$[Leu$^8$]bradykinin (30 μM), but not by the bradykinin $B_2$ receptor antagonist (Hock et al., 1991) D-Arg [Hyp$^3$, Thi$^5$, D-Tic$^7$, Oic$^8$]bradykinin (HOE140; 10 μM) (Fig. 2B). Together, these experiments imply that the [$^3$H]des-Arg$^{10}$-kallidin binding sites formed in response to interleukin-1β were fully functional bradykinin $B_1$ receptors.

The time-course of the effect of interleukin-1β on bradykinin $B_1$ receptor expression was investigated at both the mRNA and protein level using Northern blot analysis in parallel with binding studies. Total RNA samples extracted from control fibroblasts and fibroblasts that had been treated with interleukin-1β (100 IU/ml) were hybridised with a probe for the bradykinin $B_1$ receptor. This probe revealed a 1.6-kb band, which is in good agreement with the size of the human bradykinin $B_1$ receptor mRNA reported by Menke et al. (1994). Interleukin-1β caused a rapid (by 30 min) and significant ($P < 0.05$, Student's *t*-test) increase in bradykinin $B_1$ receptor mRNA that peaked between 2 and 4 h and remained significantly elevated for at least 20 h in the continued presence of interleukin-1β (Fig. 3B). Phosphorimager analysis of the 2 and 4 h bands on the blots indicated that the level of

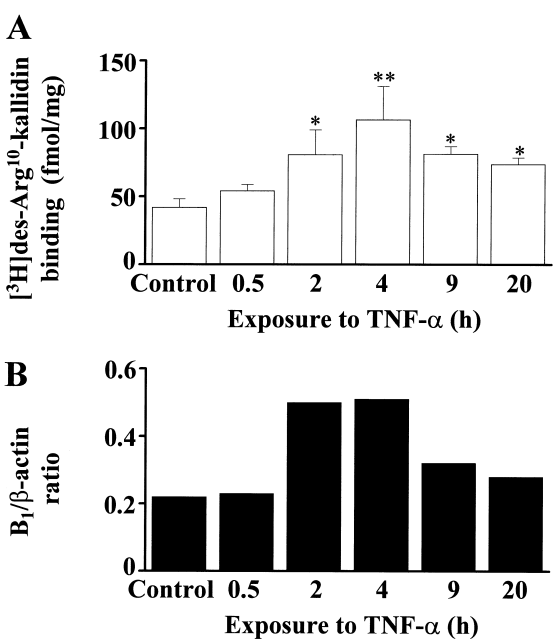


Fig. 4. Time-course of the effect of TNF-α on $B_1$ mRNA levels and [$^3$H]des-Arg$^{10}$-kallidin binding in WI-38 cells. The cells were treated with TNF-α (350 RU/ml) for the times as indicated. (A) Specific [$^3$H]des-Arg$^{10}$-kallidin binding in WI-38 cells. The data represent the means ± sem of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ Student's *t*-test compared to specific binding in control cells. (B) Phosphorimager quantitation for $B_1$ mRNA signals calculated as the ratio of β-actin mRNA. The data represent the means of two independent experiments which gave similar results.

bradykinin $B_1$ receptor mRNA had increased by a factor of 9–10 ($P < 0.01$, Student's *t*-test) compared to the mRNA extracted from untreated WI-38 cells (Fig. 3B,C).

Exposure of the cells to interleukin-1β (100 IU/ml) for 30 min had no effect on the specific binding of [$^3$H]des-Arg$^{10}$-kallidin, but the binding had increased following a 2-h exposure to the cytokine (Fig. 3A). The peak expression of bradykinin $B_1$ receptor protein occurred at 4 h, when the level of [$^3$H]des-Arg$^{10}$-kallidin specific binding was three times higher than the control level ($P < 0.01$, Student's *t*-test), and it was still significantly above the baseline ($P < 0.01$, Student's *t*-test) after 20 h (Fig. 3A).

In contrast to the rapid and substantial rise in bradykinin $B_1$ receptor mRNA, there was a smaller, three- to fourfold increase in the intensity of the 4.4-kb mRNA encoding the bradykinin $B_2$ receptor in the interleukin-1β-treated cells (Fig. 3). This increase only became significant ($P < 0.05$, Student's *t*-test) after a 4-h exposure to interleukin-1β. $B_2$ receptor mRNA remained elevated for a further 16 h (Fig. 3). There was also a small increase in [$^3$H]bradykinin-specific binding in cells exposed to interleukin-1β for 9 and 24 h ($52 \pm 6\%$ ($n = 6$) above control, and $83 \pm 7\%$ ($n = 6$) above control), but no change was observed at earlier times.

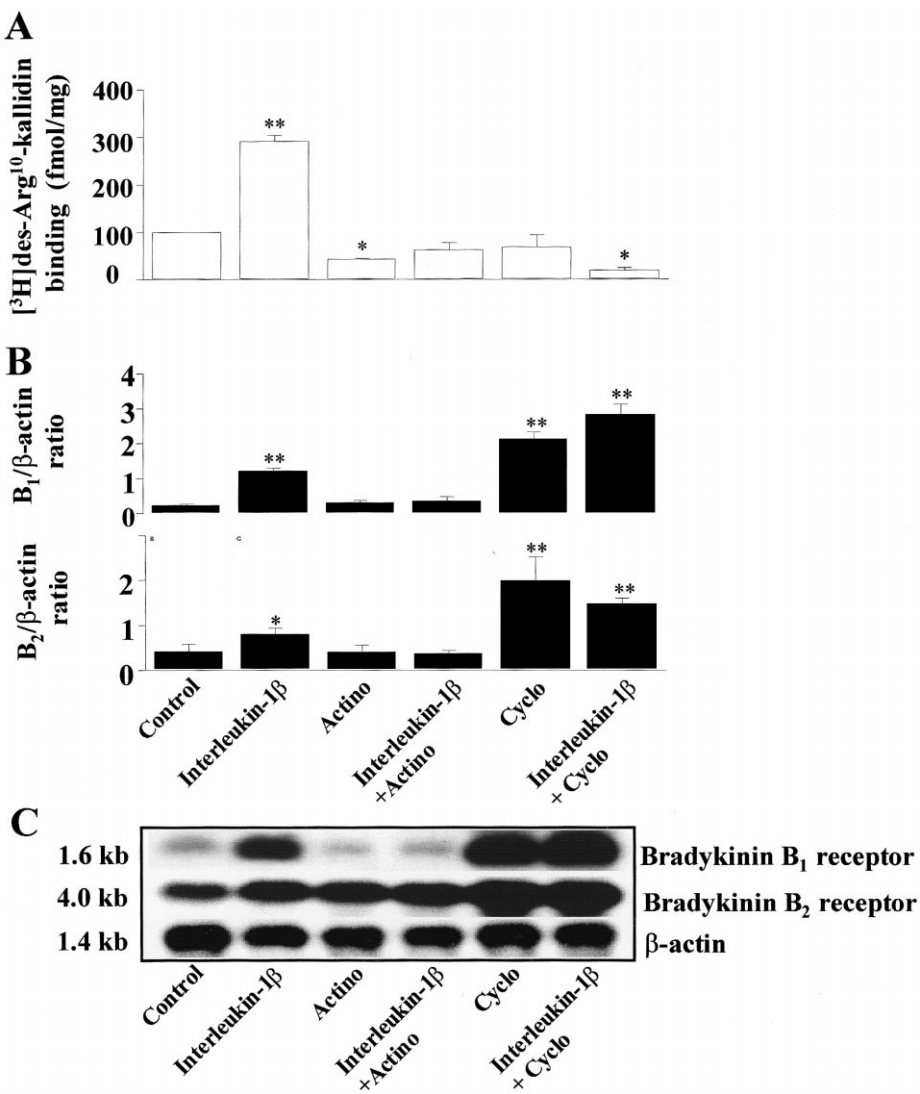


Fig. 5. Effect of cycloheximide and actinomycin D on $B_1$ and $B_2$ mRNA levels and on [$^3$H]des-Arg$^{10}$-kallidin binding in control and interleukin-1β-treated WI-38 cells. WI-38 cells were pre-treated for 1 h with cycloheximide (Cyclo, 70 μM) or actinomycin D (Actino, 5 μg/ml) in the presence or absence of interleukin-1β (100 IU/ml) for 4 h. (A) Specific [$^3$H]des-Arg$^{10}$-kallidin (1 nM) binding in WI-38 cells. The data represent the means ± sem of three independent experiments. (B) Phosphorimager quantitation for $B_1$ mRNA (top panel) and $B_2$ mRNA signals (bottom panel) calculated as the ratio of β-actin mRNA signal. The data represent the means ± sem of three independent experiments. (C) Representative Northern blot for $B_1$ mRNA (top panel), $B_2$ mRNA (middle panel) and β-actin mRNA (bottom panel) is shown from one of three experiments, which gave similar results. $^{*}P < 0.05$, $^{**}P < 0.01$ Student's *t*-test compared to untreated cells.

### 3.2. Effect of TNF-α on bradykinin $B_1$ and $B_2$ receptor expression

Exposure of WI-38 cells to TNF-α (350 RU/ml) also increased bradykinin $B_1$ receptor mRNA and binding (Fig. 4). Bradykinin $B_1$ receptor mRNA increased by 2 h, remained elevated at 4 h and declined towards basal levels by 9 h. The twofold increase in bradykinin $B_1$ receptor mRNA, which occurred between 2 and 4 h, was reflected by a twofold increase in [$^3$H]des-Arg$^{10}$-kallidin binding, which peaked at 4 h (Fig. 4) and remained significantly elevated ($P < 0.05$, Student's *t*-test) for at least 20 h. In contrast to interleukin-1β, treatment of the cells with TNF-α (350 RU/ml) for up to 20 h failed to increase either bradykinin $B_2$ receptor mRNA or [$^3$H]bradykinin binding (data not shown).

Since sites of inflammation contain elevated levels of both interleukin-1β and TNF-α, the effect of a combination of maximally effective concentrations of interleukin-1β (100 IU/ml; Zhou et al., 1998) and TNF-α (350 RU/ml; Ni et al., 1998; Promega) on $B_1$ expression was also examined. A 4-h exposure of the cells to interleukin-1β plus TNF-α caused a two- to threefold increase in [$^3$H]des-Arg$^{10}$-kallidin binding that was similar in size to the increases observed with each of the two cytokines alone (data not shown). This result suggests that both interleukin-1β and TNF-α may act via shared pathways to increase bradykinin $B_1$ receptor expression.

### 3.3. Mechanism of interleukin-1β-induced upregulation of bradykinin $B_1$ and $B_2$ receptors WI-38 cells

The requirement for protein synthesis for the upregulation of [$^3$H]des-Arg$^{10}$-kallidin and [$^3$H]bradykinin binding in WI-38 cells stimulated with interleukin-1β was investigated using the protein synthesis inhibitor cycloheximide. Cycloheximide (70 μM for 1 h; Fig. 5A) prevented the increase in [$^3$H]des-Arg$^{10}$-kallidin binding sites induced by interleukin-1β, reducing binding from 291 ± 12% of control ($n = 3$) in the presence of interleukin-1β to 19 ± 5% of control ($n = 3$) after treatment with interleukin-1β plus cycloheximide. Thus, treatment with cycloheximide plus interleukin-1β reduced [$^3$H]des-Arg$^{10}$-kallidin binding to a level that was lower than the level in untreated cells and cells that had been treated with cycloheximide alone. This suggests that interleukin-1β may be causing an increase in the rate of bradykinin $B_1$ receptor degradation as well as synthesis. Fig. 5 also shows that cycloheximide actually increased the density of the 1.6-kb band corresponding to the bradykinin $B_1$ receptor mRNA. The density of this band in cells treated with a combination of cycloheximide and interleukin-1β was no greater than in cells treated with cycloheximide alone (Fig. 5B,C). Incubation of cells with interleukin-1β and the transcription inhibitor actinomycin D completely prevented the interleukin-1β-mediated increases in bradykinin $B_1$ receptor mRNA and [$^3$H]des-Arg$^{10}$-kallidin binding (Fig. 5A,B). Qualitatively, similar results were obtained when the effect of cycloheximide and actinomycin D on the interleukin-1β-mediated increase in $B_2$ receptor mRNA was investigated (Fig. 5B,C).

## 4. Discussion

Tissue damage and inflammation leads to an increase in levels of proinflammatory cytokines including interleukin-1β and TNF-α (Schulze-Koops et al., 1997). Interleukin-1β induces the expression of bradykinin $B_1$ receptors in vitro (Galizzi et al., 1994; Marceau, 1995; Phagoo et al., 1997; Zhou et al., 1998) and in vivo (Davis and Perkins, 1994), but the importance of the related cytokine, TNF-α, in bradykinin $B_1$ receptor upregulation is not clear. This study provides evidence that like interleukin-1β, TNF-α is capable of promoting the upregulation of bradykinin $B_1$ receptor expression (Phagoo et al., 1997; Ni et al., 1998). We have previously shown that WI-38 fibroblasts express some bradykinin $B_1$ receptors constitutively and that they express approximately three times as many bradykinin $B_2$ receptors (Phagoo et al., 1996). The present study shows that both bradykinin $B_1$ and $B_2$ receptor expression can be increased by exposing the WI-38 cells to interleukin-1β. The observed increase in specific binding of [$^3$H]des-Arg$^{10}$-kallidin was due to the synthesis of bradykinin $B_1$ receptor protein rather than to a shift in the equilibrium constant for the radioligand as there was a significant increase in the $B_{max}$ value with no significant change in the $K_D$ value. Increases in the abundance of bradykinin $B_1$ receptors, without any change in affinity have also been reported in rabbit aorta following treatment with interleukin-1β, lipopolysaccharide or epidermal growth factor (Galizzi et al., 1994; Schneck et al., 1994; Levesque et al., 1995).

Activation of kinin receptors results in mobilisation of intracellular calcium stores (Smith et al., 1995; Mathis et al., 1996) and the [$^3$H]des-Arg$^{10}$-kallidin binding sites induced by interleukin-1β were functional receptors with a pharmacological profile consistent with bradykinin $B_1$ receptors.

The kinetics of the interleukin-1β-induced upregulation of the bradykinin $B_1$ receptor in the WI-38 fibroblasts was consistent with those obtained in animal models of inflammation and in isolated organ preparations. The response to bradykinin $B_1$ receptor agonists in preparations such as the rabbit aorta is not observed until 1 or 2 h after isolation and continues to develop for up to 12 h. Although isolated tissues are capable of producing interleukin-1β (Clinton et al., 1991), this process can be accelerated by exogenous interleukin-1β (Marceau, 1995). In vivo, the bradykinin $B_1$ receptor agonist, des-Arg$^9$-bradykinin is ineffective unless there has been an inflammatory insult. For example, in models of inflammatory hyperalgesia (Davis and Perkins, 1994), responsiveness to bradykinin $B_1$ receptor agonists

develops within 1 h of an inflammatory insult such as carrageenan or Freund's adjuvant and can last for over 3 days. Following exogenous administration of interleukin-1β, $B_1$-mediated hyperalgesia also develops within 1 h and can last for at least over 24 h (Davis and Perkins, 1994).

Although interleukin-1β increased $B_2$ mRNA and protein in the WI-38 cells, the effect was less pronounced than its effect on the bradykinin $B_1$ receptor. It has previously been reported that bradykinin $B_2$ receptors can be upregulated by cytokines, including interleukin-1β (Bathon et al., 1992; Angel et al.,1994; Schmidlin et al., 1998) and other factors such as activated Ras, platelet-derived growth factor (PDGF) and cAMP-elevating agents (Dixon, 1994; Dixon et al., 1996; Hembree and Leeb-lundberg, 1996). The increase in bradykinin $B_2$ receptor density was also relatively modest in these studies. We note that basal bradykinin $B_2$ receptor expression is much higher than the bradykinin $B_1$ receptor in WI-38 cells and there is, therefore, less scope for dramatic upregulation.

The production of TNF-α, which is released during inflammation (Cunha et al., 1992) is induced by kinins (Tiffany and Burch, 1989; Ferreira et al., 1993). Although interleukin-1β can induce $B_1$-mediated hyperalgesia (Davis and Perkins, 1994; Perkins and Kelly, 1994; Marceau, 1995), the role of TNF-α remains unclear. Studies, which showed a role for interleukin-1β in $B_1$-mediated hyperalgesia, failed to show any effect of TNF-α (Davis and Perkins, 1994; Perkins and Kelly, 1994). We have previously suggested that TNF-α can upregulate bradykinin $B_1$ receptors (Phagoo et al., 1997), and this was confirmed in the current study. TNF-α increased bradykinin $B_1$ receptor mRNA and [$^3$H]des-Arg$^{10}$-kallidin binding in WI-38 cells to the same extent as interleukin-1β. Indeed, the combination of maximally effective concentrations of TNF-α and interleukin-1β produced a similar increase in bradykinin $B_1$ receptor expression as the individual effects of the two cytokines, suggesting they may use a common mechanism. It is possible, however, that the mechanism of bradykinin $B_1$ receptor upregulation in inflammatory hyperalgesia is not the same as in WI-38 cells. In the WI-38 cells, interleukin-1β and TNF-α did differ in that only interleukin-1β upregulated the bradykinin $B_2$ receptor.

In order to determine whether de novo synthesis of receptor protein was responsible for the interleukin-1β-induced increase in $B_1$ expression, WI-38 cells were treated with interleukin-1β in the presence of the protein synthesis inhibitor cycloheximide or the transcription inhibitor actinomycin D. Cycloheximide itself caused a significant increase in bradykinin $B_1$ receptor mRNA levels in the WI-38 cells. Early response genes that do not require preceding protein synthesis are often superinduced after treatment with cycloheximide and Zhou et al. (1998) have previously shown cycloheximide-mediated upregulation of human bradykinin $B_1$ receptor mRNA occurs as a result of an increase in mRNA stability. Interleukin-1β did not cause a further increase in bradykinin $B_1$ receptor mRNA in the presence of cycloheximide. One explanation for this, may be that, like cycloheximide, interleukin-1β increases mRNA levels via mRNA stabilisation and not through an increase in the rate of transcription. However, in vascular smooth muscle cells, Ni et al. (1998) have shown that interleukin-1β increases bradykinin $B_1$ receptor mRNA levels via a transcriptional mechanism. It has also been reported that bradykinin $B_1$ receptor induction promoted by interleukin-1β occurs through activation of nuclear factor kappa-B (Ni et al., 1998; Schanstra et al., 1998). Our results indicate that the de novo synthesis of protein factors is not required for bradykinin $B_1$ receptor mRNA upregulation.

Actinomycin D prevented the increase in bradykinin $B_1$ receptor mRNA and in [$^3$H]des-Arg$^{10}$-kallidin binding sites. This could be due to inhibition of interleukin-1β-induced transcription. Alternatively, if the increase in steady-state mRNA levels induced by interleukin-1β is due to mRNA stabilisation, as suggested by the experiments with cycloheximide, then the effect of actinomycin D may be due to inhibition of basal transcription.

The more modest interleukin-1β-induced upregulation of $B_2$ mRNA and protein was also prevented by actinomycin D. Cycloheximide blocked the increase in [$^3$H]bradykinin binding sites and enhanced the increase in bradykinin $B_2$ receptor mRNA. In contrast to bradykinin $B_1$ receptors, there is no evidence that bradykinin $B_2$ receptors are regulated during inflammation. However, short-term decreases in cell surface receptor density and desensitisation after receptor activation have been reported (Roberts and Gullick, 1990). This is in contrast to bradykinin $B_1$ receptors, which do not exhibit profound desensitisation and appear not to internalise after exposure to agonists (Smith et al., 1995; Mathis et al., 1996; Austin et al., 1997). In some systems, it has been reported that activation of bradykinin $B_2$ receptors can contribute to upregulation of bradykinin $B_1$ receptors, suggesting interaction between the two receptor subtypes where they occur together (Phagoo et al., 1999).

Our results show that bradykinin $B_1$ receptors are upregulated in WI-38 fibroblasts by the pro-inflammatory cytokines interleukin-1β and TNF-α. The presence of inflammatory cytokines primes the site of injury for inflammation and may act with kinins to perpetuate a feed-forward mechanism of inflammation. This may be an explanation for the co-expression of two receptor subtypes that function through similar, if not identical, pathways. The bradykinin $B_2$ receptor is expressed in abundance and undergoes rapid desensitisation and internalisation (Roberts and Gullick, 1990), which is compensated by a modest upregulation of receptor sites by interleukin-1β. On the other hand, bradykinin $B_1$ receptors, which are initially expressed at low levels because of rapid mRNA turnover (Zhou et al., 1998) are upregulated many-fold and are not functionally downregulated by desensitisation or internalisation.

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