Roles for interleukin-1β, phorbol ester and a post-transcriptional regulator in the control of bradykinin B1 receptor gene expression

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Bradykinin B1 receptor (BKB1R) is involved in a variety of pathophysiological processes, particularly those related to inflammation. The gene for this receptor is known to be upregulated by interleukin (IL)-1β, a proinflammatory cytokine. However, the molecular mechanisms involved in the regulation of the BKB1R gene expression have not been defined. We demonstrated that IL-1β induces a rapid increase in BKB1R mRNA level and the binding of desArg^{10}-kallidin in human embryo lung fibroblasts (IMR90). This increase in BKB1R mRNA level is protein synthesis-independent as indicated by treatment of cells with cycloheximide (CHX) or puromycin (PUR). By testing the IL-1β effect on BKB1R mRNA degradation, we showed that the IL-1β upregulation of BKB1R expression is achieved through both transcriptional activation and post-transcriptional mRNA stabilization. In addition to the IL-1β effects, translation inhibitors, CHX and PUR increase the steady state BKB1R mRNA level by inhibiting BKB1R mRNA degradation. Removal of the CHX block with subsequent resumption of protein synthesis results in a sizable increase of desArg^{10}-kallidin binding. Using signalling pathway inhibitors, we show that IL-1β functions through a protein tyrosine kinase, not protein kinase C or protein kinase A. However, activation of protein kinase C by phorbol 12-myristate 13-acetate increases the level of BKB1R mRNA and the binding of desArg^{10}-kallidin. This increase is blocked by NF-κB activation inhibitors.

INTRODUCTION

Strong evidence links bradykinin and related kinin effectors such as desArg^{9}-BK and desArg^{10}-kallidin with pathophysiological processes that accompany inflammation and tissue damage, including the induction of pain and hyperalgesia [1]. Two principal BK receptor types, B1 and B2, have been reported [2]. The bradykinin B1 receptor (BKB1R) gene is induced rapidly by inflammatory processes and bacterial infection [1]. Interleukin-1β (IL-1β), which characteristically upregulates inflammation related gene expression such as cyclooxygenase-2 (COX2) [3] and inducible nitric oxide synthase (iNOS) [4], also rapidly upregulates BKB1R expression [5,6]. Human cDNA as well as the entire gene, including the promoter region of this receptor, have been cloned and characterized [5,7]. However, the mechanisms regulating BKB1R gene expression have not been reported to date. Understanding these mechanisms is important not only with regard to this gene but also other genes involved in the inflammatory cascade displaying similar, rapid responses to inflammatory agents such as IL-1β.

Our results show that multiple mechanisms regulate the expression of this gene. These include both transcriptional and post-transcriptional mechanisms involving multiple signalling paths.

EXPERIMENTAL

Materials

Human recombinant IL-1β was purchased from Genzyme (Cambridge, MA); Actinomycin D (AMD), cycloheximide (CHX), puromycin (PUR), phorbol 12-myristate 13-acetate (TPA), genistein and pyrrolidinedithiocarbamate (PDTC) were purchased from Sigma; 2′-Amino-3′-methoxyflavone (PD-98059), *N*-[2-((*p*-bromocinnamyl)amino-)ethyl]-5-isoquinolinesulphonamide, HCl (H-89) and α-cyano-(3-hydroxy-4-nitro)cinnamonitrile (tyrphostin AG-126) were purchased from Calbiochem (La Jolla, CA).

Cell culture

Human embryo lung fibroblasts, IMR90 were obtained from Coriell Institute for Medical Research (Camden, NJ). The cells were maintained in minimal essential (MEM) medium containing 10 % foetal bovine serum (FBS) and 50 units/ml penicillin, 50 μg/ml streptomycin. The cells were used at population doubling level 18–25. Prior to experimentation, the cells were preincubated in MEM containing 0.4 % FBS for 18 h and then stimulated with or without the test agents for the time indicated.

Specific binding

The binding assay was modified from a protocol described previously [8]. Briefly, cells in 24-well plates were washed three times with 1 ml of PBS at 4 °C followed by a 15 min equilibration with 0.2 ml of modified Hank's balanced salt solution (HBSS) with 0.05 % BSA. The binding medium was removed and replaced with fresh, chilled medium containing 10 nM [^{3}H]desArg^{10}-kallidin (DuPont–NEN). Non-specific binding was determined in the presence of 3 μM desArg^{10}-kallidin. After a 2 h incubation at 4 °C, the medium was aspirated and the cells were washed four times with 1 ml of the HBSS with 0.2 % BSA. Bound radioactivity was determined by solubilizing the cells in 0.5 ml of 0.2 % SDS and quantification in a Pharmacia Biotech Inc. liquid scintillation β counter. All determinations were done in triplicate.

Abbreviations used: BKB1R, bradykinin B1 receptor; IL-1, interleukin-1; CHX, cycloheximide; PUR, puromycin; TPA, phorbol 12-myristate 13-acetate; PKA, protein kinase A; PTK, protein tyrosine kinase; AMD, actinomycin D; PDTC, pyrrolidinedithiocarbamate.

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Northern blot analysis

Total RNA was extracted from cells by the guanidinium thiocyanate-phenol-chloroform procedure according to the methods described previously [8]. RNA was quantitated by ultraviolet absorbance at 260 nm, and 10 μg denatured RNA was electrophoresed on a 1 % agarose/formaldehyde denaturing gel and transferred to nylon membranes (DuPont–NEN). The blots were hybridized sequentially with ^{32}P-labelled cDNA probes, BKB1R and ubiquitin, at 65 °C in a Rapid-hyb buffer (Amersham Life Science, Amersham, U.K.) and washed as described [8]. Radioactivity associated with each band was quantified by autoradiography with an intensifying screen at −80 °C and with an Instantimager, an electronic autoradiography instrument (Packard, Meriden, CT). All incubations for Northern blots were done in duplicate. The cDNA probe for BKB1R was produced as follows: total RNA from IMR90 cells, treated with IL-1β for 2 h was converted to single-strand cDNA by reverse transcription, then amplified by PCR with 5′-CTGTGCATGGCATCATCCTGGCCC-3′ as sense prime and 5′-CAATGCTGTTTTAATTCCGCCA-3′ as antisense primer. The PCR product was subcloned into PCRII™ vector and sequenced using a plasmid double-stranded DNA sequencing kit USD (Cleveland, OH) and an ABI automatic sequencer model 373A. The cDNA was then released by digestion with *Hin*dIII and *Xba*I (New England Biolabs, Beverly, MA). The cDNA probe for ubiquitin was obtained from American Type Culture Collection (Rockville, MD).

To determine mRNA half-life, AMD (5 μg/ml) was added to cells. RNA was isolated from samples of cells taken at several time points after the addition of AMD. Northern blot analysis was performed as described above.

Nuclear run-on analysis

Nuclei isolation and nuclear transcription assays were performed as described by Greenberg and Ziff [9] with some modifications. Nuclear RNA samples were isolated with Tri-Reagent (Molecular Research Center, Cincinnati, OH). Each [^{32}P]UTP (DuPont–NEN) labelled nuclear RNA sample (2×10^6 cpm) was then hybridized to 6 μg of plasmid DNA (Puc), containing the inserts of BKB1R or ubiquitin, at 65 °C in a Rapid-hyb buffer (Amersham Life Science, Amersham, U.K.) for 18 h. Autoradiography was done with an intensifying screen at −80 °C.

[^{3}H]Leu incorporation

Protein synthesis was estimated by incorporation of [^{3}H]Leu into acid insoluble material. After treatment, cells in 96-well plates were incubated with [^{3}H]Leu (180 Ci/mmol, DuPont–NEN) for 2 h. Cells were then washed three times and 60 μl of 0.4 M KOH and 140 μl of 10 % trichloroacetic acid (TCA) was added. The acid insoluble fraction was separated by a mesh-harvester and radioactivity associated with the acid insoluble fraction was quantified in a Pharmacia Biotech Inc. liquid scintillation β counter.

RESULTS

IL-1β induced expression of BKB1R gene

Binding of desArg^{10}-kallidin, a specific agonist for the BKB1R, increases approximately 8-fold within 4 h after stimulation of IMR90 cells with 100 pg/ml IL-1β (Figure 1A). The binding then declines to 3-fold of basal levels by 24 h after IL-1β stimulation. A similar pattern of BKB1R mRNA level following IL-1β treatment is illustrated in Figure 1C. In this case maximum mRNA level is reached at 2 h post-stimulation. The mRNA levels then drop visibly by 8 h post IL-1β treatment. As illustrated in Figure 1B, maximal receptor upregulation occurs with 10 pg/ml of IL-1β and then plateaus. All subsequent studies with IL-1β were done at 100 pg/ml. As shown by nuclear run-on analysis, the transcription of BKB1R gene is increased after stimulation of the cells with IL-1β for 30 min (Figure 1D). CHX does not increase transcription.

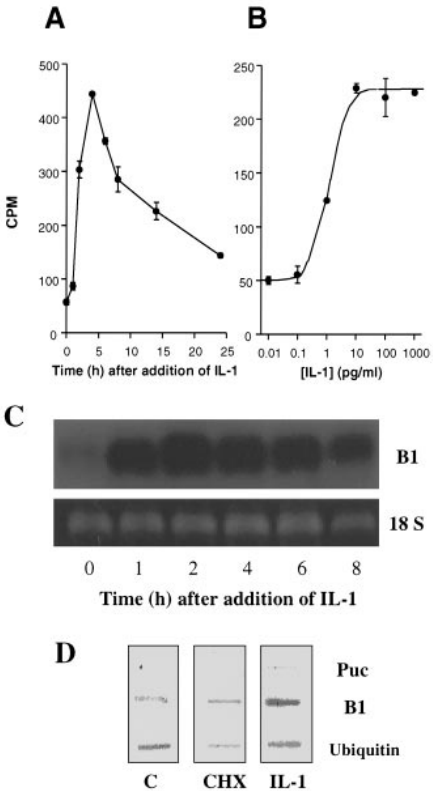

Figure 1 IL-1β induced expression of BKB1R gene

(**A**) IMR90 cells cultured in 24-well plates were treated with 100 pg/ml IL-1β for the indicated times. Binding of cells to [^{3}H]desArg^{10}-kallidin was determined as described in Methods. The data represent three separate experiments. The error bars represent standard deviation. (**B**) binding of [^{3}H]desArg^{10}-kallidin was determined in cells exposed to increasing IL-1β concentration for 4 h. The data represent three separate experiments. The error bars represent standard deviation. (**C**) Northern blot autoradiography showing mRNA concentration after treatment of IMR90 with IL-1β for 0–8 h. The formaldehyde gel for ribosomal 18S was shown to demonstrate equal loading. The Northern blot shown is one of two experiments showing similar results. (**D**) Nuclei were isolated from cells treated with vehicle, 100 pg/ml IL-1β or 10 μg/ml CHX, for 30 min. Nuclear run-on procedure was performed as described. Nuclear RNA samples were hybridized to BKB1R, ubiquitin and Puc (plasmid) cDNA. The nuclear run-on shown represents one of four identical experiments.

IL-1β and CHX induce stabilization of BKB1R mRNA

Several reports have suggested that IL-1β regulates expression of other genes at the post-transcriptional level [10,11]. To evaluate involvement of post-transcriptional mechanisms in BKB1R upregulation by IL-1β, we investigated the turnover of mRNA (Figures 2A and 2B). Cells were pretreated with IL-1β for 2 h or with CHX for 30 min and then AMD was added. RNA was isolated from the cells at several time points following the addition of AMD, and Northern blot analysis was performed as described. Under basal conditions the BKB1R transcript decayed rapidly with a half-life of approx. 1 h. The rapid degradation of the BKB1R mRNA was inhibited, somewhat, by IL-1β, which

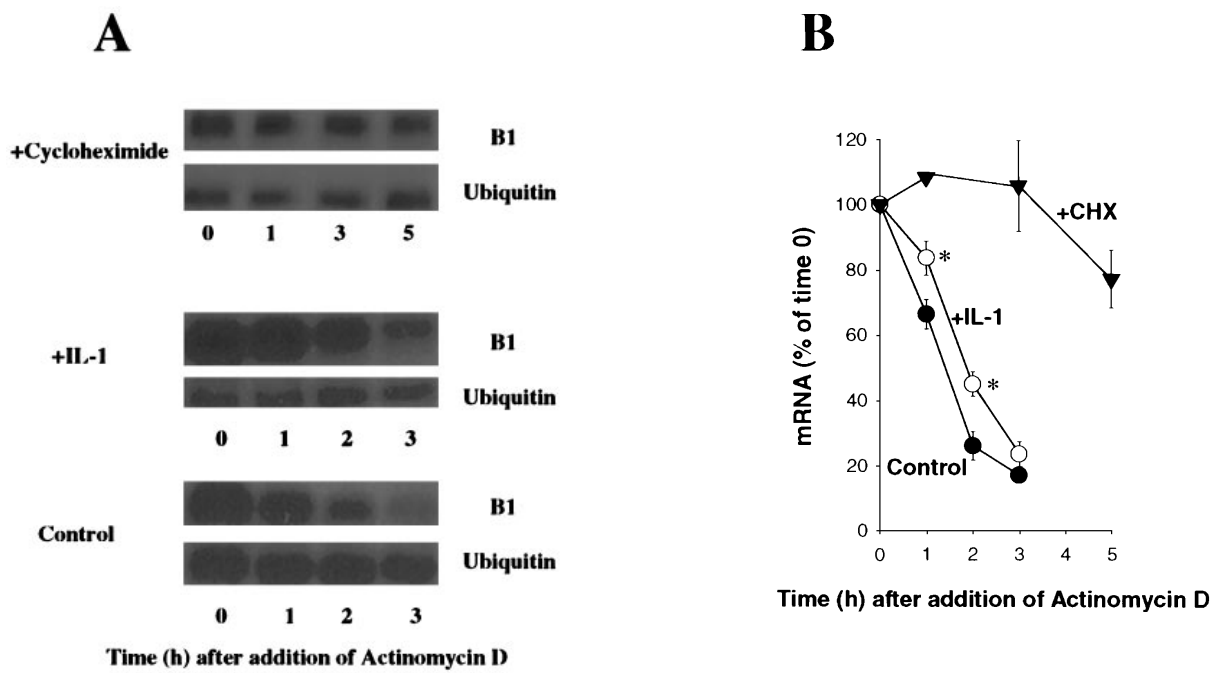

Figure 2 IL-1β and CHX effects on BKB1R mRNA stability

(**A**) IMR90 cells were pretreated with 10 μg/ml CHX for 30 min or 100 pg/ml IL-1β for 2 h. Actinomycin D (5 μg/ml) was then added to the cultures and RNA was then isolated at 0, 1, 3 and 5 h from CHX-treated cells and at 0, 1, 2 and 3 h from IL-1β and control cells. Northern blot was performed as described in the Experimental section. In order to visualize all the bands, the control group RNA was loaded with 40 μg of RNA while the rest was 10 μg on the gel. The same blot was probed for ubiquitin to demonstrate even loading. The Northern blots shown represent one of three separate experiments. (**B**) The amount of radioactivity in the bands was quantified with Instantimager and plotted as percentage of radioactivity at time 0. Data is normalized according to loading standard. The error bars represent standard deviations of triplicate experiments. Student's *t* test was used to determine significant difference between control and IL-1-treated cells. Each point was compared to corresponding control. *P* values < 0.05 (*).

extended the mRNA half-life from 1 to 2 h (Figure 2B). However, CHX, a translation inhibitor, had a much more pronounced effect on BKB1R mRNA level and extended the BKB1R mRNA half-life from 1 h to over 5 h (Figure 2B).

Effect of transcription and translation inhibitors on IL-1β-induced BKB1R mRNA

To evaluate the role of transcription and translation inhibitors on BKB1R gene expression, IMR90 cells were pretreated with 5 μg/ml AMD, 10 μg/ml CHX, 100 μg/ml PUR or vehicle alone for 30 min and then stimulated with IL-1β (Figures 3A and 3B). Pretreatment with the transcriptional inhibitor, AMD, reduced both basal and IL-1β-induced BKB1R mRNA levels, suggesting that transcriptional activation is essential for IL-1β-induced gene upregulation. However, AMD did not completely block the IL-1β effect. Instead, the IL-1β-induced increase in mRNA level was reduced from a 4-fold increase in absence of AMD to a 1.4-fold increase in its presence (Figure 3B), confirming that a post-transcriptional event is participating in the overall IL-1β effect. An additional Northern blot, with high loading (40 μg total RNA), verified the 1.4-fold increase by IL-1β in the presence of AMD (data not shown). In the presence of either CHX or PUR, both basal and IL-1β-induced BKB1R mRNA levels increased (Figure 3B). This was in agreement with the observed stabilization by CHX of BKB1R mRNA (Figure 2). Furthermore, in those cells pretreated with CHX or PUR, IL-1 treatment still caused an increase in BKB1R mRNA level. This suggests that protein synthesis is not needed for this event.

Effect of CHX on specific binding for BKB1R

To confirm the observed effect of CHX on BKB1R mRNA level, we used the reversible property of this inhibitor to free protein synthesis and generate a new receptor after removal of the CHX block. As illustrated in Figure 4(A), protein synthesis was inhibited at more than 95 % within 5 min of CHX addition. Protein synthesis was then recovered to 50 % of basal at 15 min after removal of CHX (Figure 4B). Under these conditions, we observed that the specific binding of desArg^{10}-kallidin increased 2-fold and 4-fold over basal, following the removal of CHX for 60 min and 120 min respectively (Figure 4C).

Role of protein kinase C (PKC), MAPK, NF-κB and protein kinase A (PKA) in BKB1R expression

Protein kinase C and its potential downstream signalling target, MAPK, have been reported to be involved in the IL-1β-mediated signal path [12]. To test the involvement of PKC and MAPK in the upregulation of the BKB1R gene, we examined the effect of TPA, a PKC activator, and PD98059, a MAPK kinase inhibitor [13,14], on IL-1β-mediated upregulation. IMR90 were pretreated with 20 μM PD98059 for 30 min and then stimulated with 100 pg/ml IL-1β or 100 nM TPA. Northern blot analysis was performed as described. The MAPK kinase inhibitor, PD98059, had no apparent effect on basal or IL-1β-mediated accumulation of BKB1R mRNA (Figure 5A), but decreased the TPA effect on BKB1R mRNA level. Furthermore, inactivation of PKC with high concentration (500 nM) and long exposure (18 h) to TPA [15] did not affect the IL-1β-mediated

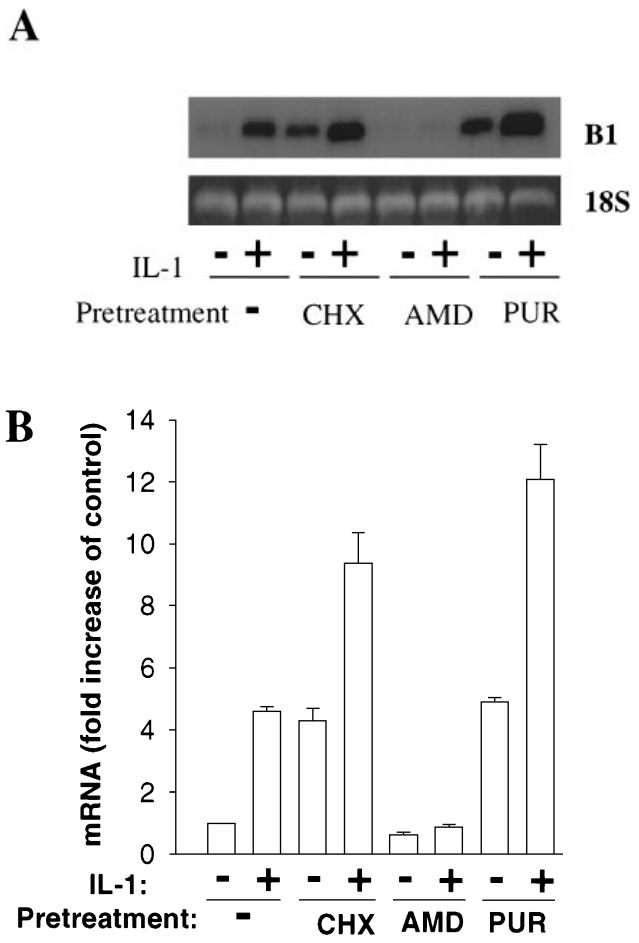

Figure 3 Effect of CHX, PUR and Actinomycin D on IL-1-induced BKB1R mRNA level

(**A**) Cells were pretreated with 10 μg/ml CHX, 5 μg/ml AMD, 100 μg/ml PUR or vehicle alone for 30 min and then exposed to 100 pg/ml IL-1β. RNA was isolated and subjected to Northern blot analysis. The Northern blot shown represents one of three separate experiments. (**B**) The amount of radioactivity in the bands was estimated by an Instantimager. The error bars represent standard deviations of triplicate experiments. An additional Northern blot with high RNA loading (40 μg) was done to clearly visualize the mRNA in the AMD pretreated cells (results not shown).

BKB1R upregulation. In fact, the fold increase of BKB1R message by IL-1β in cells treated with/without 500 nM TPA was approximately the same (Figure 5B). Since both IL-1β and PKC have been shown to function through an NF-κB element [16], we tested the effect of an NF-κB activation inhibitor [17,18], PDTC, on the IL-1β- or TPA-activated BKB1R gene expression. Cells were pretreated with 100 μM PDTC for 1 h, then stimulated with 100 pg/ml IL-1β, 100 nM TPA or vehicle alone for 2 h (Figure 5C). The inhibitor had no effect on control or IL-1β-induced BKB1R mRNA levels, but decreased the TPA-induced message, further indicating that the upregulation by IL-1β follows a pathway other than PKC activation. To verify that the TPA effect is manifested at the protein level, we tested the binding of [^{3}H]desArg^{10}-kallidin (Figure 5D). When cells were treated with 100 pg/ml IL-1β or 100 nM TPA, the binding increased approximately 8- and 4-fold respectively.

PKA has also been reported to be involved in IL-1β-mediated signalling [12]. We investigated the effect of the PKA inhibitor H-89 [19,20] with a $K_i = 48$ nM on BKB1R gene

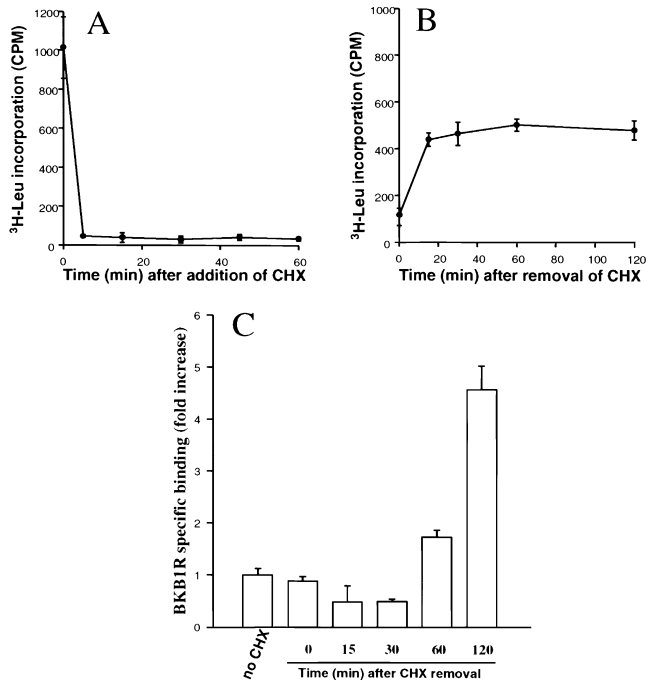

Figure 4 Reversal of inhibitory effect of CHX on protein synthesis and subsequent binding of [^{3}H]desArg^{10}-kallidin

(**A**) Cells were treated with 10 μg/ml CHX for the indicated times then labelled with [^{3}H]Leu. Incorporation of radiolabel into the acid insoluble fraction was determined as described in the Experimental section. (**B**) Cells were treated with 10 μg/ml CHX for 2.5 h then washed three times with pre-warmed medium (37 °C) to remove CHX. The [^{3}H]Leu incorporation was determined at different times after removal of CHX. (**C**) Cells were treated with 10 μg/ml of CHX for 2.5 h. Then CHX was removed by washing. Binding of [^{3}H]desArg^{10}-kallidin was determined at different times after CHX removal. The data represent two separate experiments carried out in triplicate. The error bars represent standard deviations.

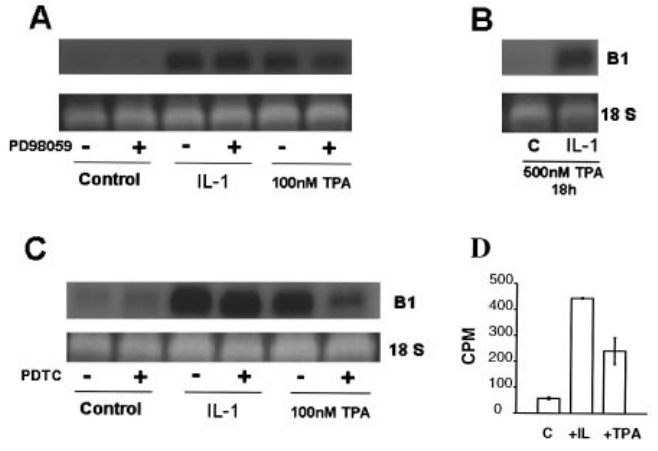

Figure 5 Effects of IL-1β and PKC activation on BKB1R expression

(**A**) Cells were pretreated with 20 μM PD-98059 or vehicle for 30 min, then stimulated with 100 pg/ml IL-1β, 100 nM TPA or vehicle alone for 2 h or (**B**) pretreated with 500 nM TPA for 18 h, then stimulated with 100 pg/ml IL-1 or vehicle for 2 h or (**C**) pretreated with 100 μM PDTC for 1 h, then stimulated with 100 pg/ml IL-1β, 100 nM TPA or vehicle alone for 2 h. The RNA was isolated and subjected to Northern blotting as described. (**D**) Cells were treated with 100 pg/ml IL-1β, 100 nM TPA or vehicle alone for 4 h, then binding of [^{3}H]desArg^{10}-kallidin was determined as in Figure 1. Each Northern blot represents one of three experiments showing similar results. The error bars in (**D**) represent standard deviation of triplicate experiments.

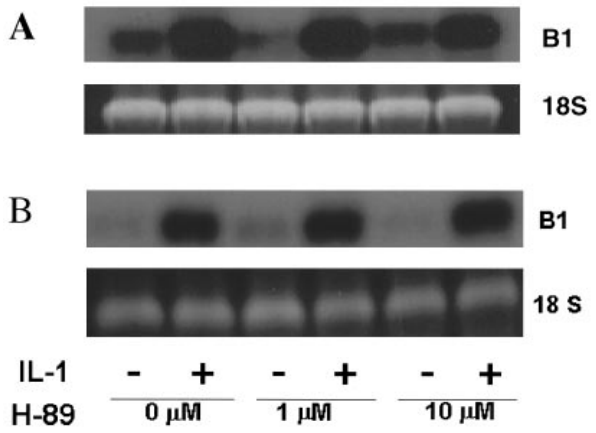

Figure 6 Effect of PKA inhibitor, H-89, on IL-1β induction of BKB1R mRNA

Cells were pretreated for 1 h with 0, 1 or 10 μM of H-89 and then stimulated with 100 pg/ml IL-1β or vehicle for 2 h. Northern blotting was carried out as described. Northern blots for two identical experiments are shown as (**A**) and (**B**).

upregulation by IL-1β. Pretreatment of IMR90 for 1 h with H-89 at 1–10 μM did not affect BKB1R expression stimulated by IL-1β and had no effect on basal expression (Figure 6). This indicates that in this case, PKA is not a signal mediator for IL-1β.

Role of protein tyrosine kinase (PTK) in IL-1β-induced BKB1R expression

Several studies link IL-1β-mediated gene upregulation with protein tyrosine kinase activity [21,22]. Two structurally and functionally different PTK inhibitors, genistein and tyrphostin AG-126 [23,24], were used to evaluate the role of these kinases in IL-1β-induced BKB1R upregulation. Genistein is a competitive inhibitor with respect to ATP binding to PTK and inhibits a wide range of PTKs [24]. At concentrations of 10–120 μM, genistein caused a dose-dependent decrease in IL-1β stimulation while the basal level BKB1R expression showed little change (Figure 7A). This suggests that a PTK is involved in the IL-1β-mediated upregulation. To further confirm a role for PTK in the IL-1β mechanism, we used another PTK inhibitor, tyrphostin AG-126. This is a synthetic PTK inhibitor which competes for the substrate binding and has been shown to block TNFα and lipopolysaccharide-induced phosphorylating events [23,24]. At concentrations of 25 and 50 μM tyrphostin AG-126 inhibited IL-1β-induced BKB1R expression in a dose-dependent fashion and had no marked effect on basal BKB1R mRNA level (Figure 7B).

DISCUSSION

Under basal culture conditions, the BKB1R gene is expressed at a low level in IMR90 cells. IL-1β induces a rapid, but transient, increase in both BKB1R mRNA and specific binding of desArg^{10}-kallidin. In the present study we demonstrate that the IL-1β-

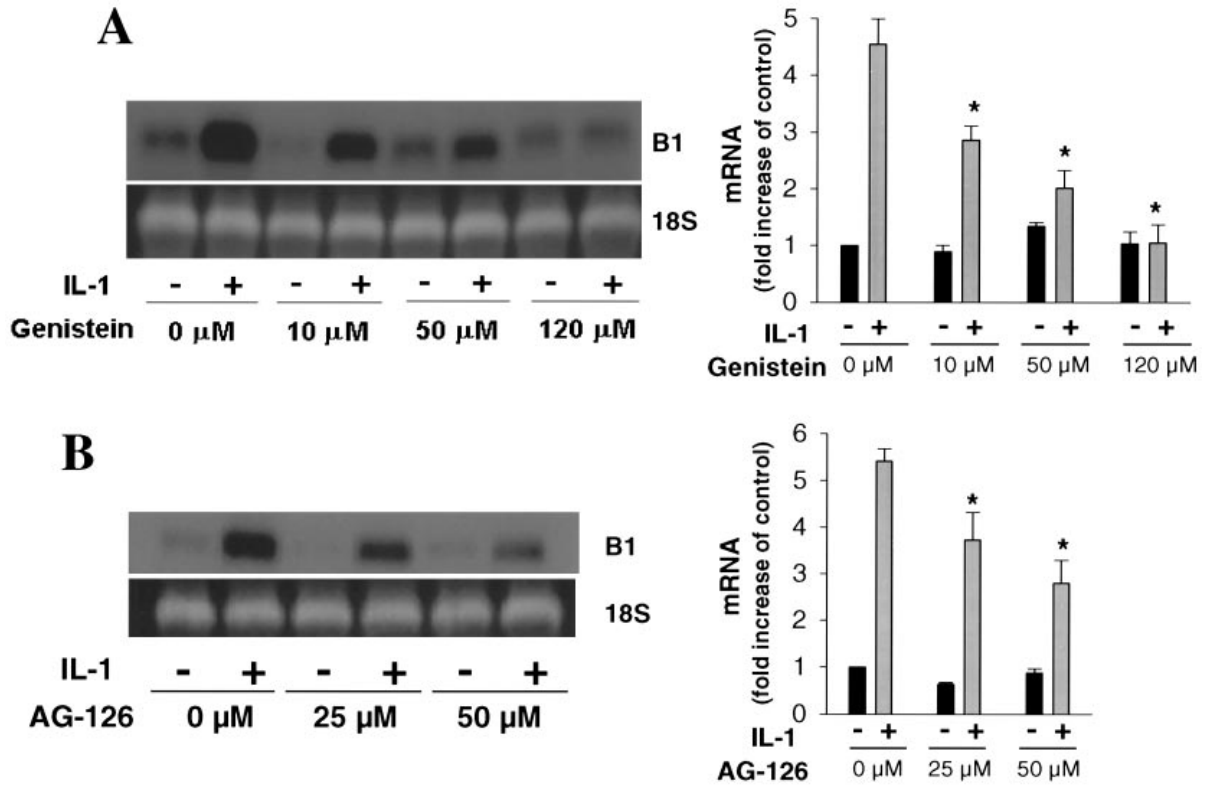

Figure 7 Effect of PTK inhibitors, genistein and tyrphostin AG-126, on IL-1β induction of BKB1R mRNA

(**A**) Cells were pretreated for 1 h with 0, 10, 50, 120 μM genistein or (**B**) pretreated for 3 h with 0, 25, 50 μM AG-126. Cells subjected to either pretreatment were then exposed to 100 pg/ml IL-1β or vehicle for 2 h. Northern blotting was carried out as described. Each Northern blot represents one of three experiments showing similar results. The amount of radioactivity in each band was determined with an Instantimager. The error bars represent standard deviation. Student's *t* test was used to determine significant difference among IL-1β-induced BKB1R mRNA levels from cells with various pretreatments. *P* values < 0.05 (*).

induced BKB1R upregulation is achieved, in part, by stabilizing the mRNA. IL-1β apparently induces some BKB1R mRNA stabilization. However the IL-1β-induced stabilization is not dramatic. It only increases the half-life of BKB1R mRNA by approximately 2-fold. This mechanism alone is not sufficient to achieve the rapid increase in mRNA observed with IL-1β treatment. Indeed, by pretreating the cells with AMD, we find that IL-1β-related post-transcriptional regulation achieves less than 0.5-fold increase in BKB1R expression, while the combined transcriptional and post-transcriptional regulation achieves a 4- to 6-fold increase of BKB1R expression.

We find that two functionally distinct translation inhibitors, CHX and PUR, increase steady-state BKB1R mRNA levels. Furthermore, we observe that the degradation of BKB1R mRNA is inhibited dramatically by CHX (Figure 2). The most likely effect of these inhibitors is to block the production of a rapidly turning-over protein which, under basal cell conditions, maintains BKB1R mRNA at a low level. Interestingly, CHX has also been reported to potentiate other rapidly induced inflammation-related genes such as iNOS and COX2 [10,25]. In fact, mRNA stability of a number of rapidly induced genes may prove to be under tight control by a class of putative, rapidly turning-over proteins [26]. With regard to the BKB1R gene, more studies are necessary to determine in what manner their presence is regulated. A potential mRNA destabilizing element, AUUUA, has been reported to exist in the 3′ untranslated regions of a number of rapidly turning-over mRNAs including those for COX2 [27] and iNOS genes [28]. This motif is also found in the BKB1R 3′ untranslated region [7].

Both CHX and PUR fail to block the IL-1β-induced increase in mRNA, illustrating that new protein synthesis is not involved in this cascade, which indicates that BKB1R is an early response gene for IL-1β. By inactivation of PKC and the use of PKA inhibitors, we find that the IL-1β signal does not function through either enzyme. Nevertheless, activation of PKC does upregulate BKB1R gene expression and this upregulation is blocked by MAPK kinase and NF-κB activation inhibitors. The IL-1β-induced BKB1R upregulation is not blocked by these two inhibitors. Instead, two structurally and functionally unrelated PTK inhibitors, genistein and tyrphostin AG-126, inhibit the IL-1β induction in a dose-dependent fashion. These observations point to transduction of the IL-1β signal via a tyrosine-phosphorylating step. Interestingly, a recent finding suggests that IL-1β signalling may be associated with the JAK-STAT path [29], a common pathway for many other cytokine signalling events [30]. It has yet to be determined if JAK is the PTK involved in our system.

Our data thus point to the existence of an IL-1β-regulated mRNA stabilization mechanism and a transcriptional level mechanism for the BKB1R gene. A putative short-lived protein appears to maintain a low basal BKB1R mRNA level through a destabilization mechanism, suggesting that under basal conditions constitutive BKB1R mRNA is produced continuously with this factor playing an important regulatory role. Both PTK and PKC appear to participate in separate processes to upregulate BKB1R transcriptional activity. It is not clear at this time if either or both paths also regulate the production or action of the putative protein. Although the induction of other genes by IL-1β has been linked to the NF-κB element, in this case it is activation of PKC which leads to BKB1R upregulation through NF-κB; whereas, IL-1β is clearly acting through a PTK pathway to transiently increase mRNA levels.

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