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**HAL Id: hal-02502598**

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Submitted on 16 Mar 2020

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*Am J Physiol Renal Physiol* 274:532-540, 1998.

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# B<sub>2</sub> kinin receptor upregulation by cAMP is associated with BK-induced PGE<sub>2</sub> production in rat mesangial cells

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**Marin Castaño, Maria E., Joost P. Schanstra, Christophe Hirtz, João B. Pesquero, Christiane Pecher, Jean-Pierre Girolami, and Jean-Loup Bascands.** B<sub>2</sub> kinin receptor upregulation by cAMP is associated with BK-induced PGE<sub>2</sub> production in rat mesangial cells. *Am. J. Physiol.* 274 (*Renal Physiol.* 43): F532–F540, 1998.—In the rat mesangial cell (MC), activation of the bradykinin B<sub>2</sub> receptor (B<sub>2</sub>R) by bradykinin (BK) is associated with both phospholipase C (PLC) and A<sub>2</sub> (PLA<sub>2</sub>) activities and with inhibition of adenosine 3',5'-cyclic monophosphate (cAMP) formation leading to cell contraction. Because cAMP plays an important role in the regulation of gene expression in general, we investigated the effect of increasing the intracellular cAMP concentration ([cAMP]<sub>i</sub>) in mesangial cells on the B<sub>2</sub> mRNA expression, on the density of B<sub>2</sub> receptor binding sites, on the BK-induced increase in both the free cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>), and in the prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production. Forskolin, PGE<sub>2</sub>, and cAMP analog, 8-bromo-adenosine 3',5'-cyclic monophosphate (8-BrcAMP), were used to increase [cAMP]<sub>i</sub>. Twenty-four-hour treatment with forskolin, PGE<sub>2</sub>, and 8-BrcAMP resulted in significant increases in B<sub>2</sub> receptor binding sites, which were inhibited by cycloheximide. The maximum B<sub>2</sub> receptor mRNA expression (160% above control) was observed in cells treated during 24 h with forskolin and was prevented by actinomycin D. In contrast, the D-myo-inositol 1,4,5-trisphosphate (IP<sub>3</sub>) formation and the BK-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>, reflecting activation of PLC, were not affected by increased levels of [cAMP]<sub>i</sub>. However, the BK-induced PGE<sub>2</sub> release, reflecting PLA<sub>2</sub> activity, was significantly enhanced. These data bring new information regarding the dual signaling pathways of B<sub>2</sub> receptors that can be differentially regulated by cAMP.

bradykinin; B<sub>2</sub> kinin receptor messenger ribonucleic acid; prostaglandin E<sub>2</sub>; free cytosolic calcium; adenosine 3',5'-cyclic monophosphate; mesangial cell

THE NONAPEPTIDE BRADYKININ (BK) is a potent vasoactive peptide generated by the proteolytic action of serine proteases called kallikreins, acting on protein precursors named kininogens, present in plasma and tissue (6). In plasma, the half-life of kinins is 15–20 s, and their concentrations in biological fluids are in the femtomolar range, far below the affinity of any identified bradykinin receptor. Therefore, it is likely that BK has to be generated very close to its site of action. Pharmacological studies have demonstrated that kinins exert their biological effects through the activation of at least two receptors, named the B<sub>1</sub> and B<sub>2</sub> receptors (49). The cloning of both receptors has revealed that they belong to the family of seven-transmembrane domain, G protein-coupled receptors. The cDNA coding

for the B<sub>1</sub> receptor has been recently cloned and characterized in rabbit, human, and mouse (32, 41, 48). The B<sub>2</sub> receptor cDNA has been cloned from rat (39), mouse (40), and human (24), and the amino acid sequence of the latter was found to be 36% identical to that of the human B<sub>1</sub> receptor. Under physiological conditions, most of the biological effects of kinins are mediated through the B<sub>2</sub> receptor. Indeed, the B<sub>1</sub> receptor is not expressed at significant levels under these conditions, but its expression is strongly induced by cytokines (35). Kinins are involved in a wide range of functions, including systemic and local hemodynamic regulation (regulation of systemic blood pressure and organ blood flow), inflammatory responses, water and electrolyte transport, and pain-transmitting mechanisms (37). BK receptors are expressed at various sites of the nephron and are involved in different effects. In renal microvessels, BK is involved in the regulation of papillary blood flow (37). In the distal collecting tubule, BK is able to reduce the action of arginine vasopressin (AVP) on water reabsorption by inhibiting the AVP-induced increase in cAMP production (37). More recently, we demonstrated the presence of BK receptors in freshly isolated glomeruli and cultured mesangial cells, which are contractile cells and represent about one-third of the glomerular cell population (18). Furthermore, we have reported that the activation of the B<sub>2</sub> receptor on cultured mesangial cells stimulates cell proliferation (5). In mesangial cells, the activation of the B<sub>2</sub> receptor by BK induces the phospholipase C (PLC) and A<sub>2</sub> (PLA<sub>2</sub>) pathways leading to cell contraction (3), but it also inhibits cAMP formation (4).

Because mesangial cells express both B<sub>1</sub> and B<sub>2</sub> receptors and may be involved in opposite effects, it is of great interest to define more precisely some aspects of the regulation of B<sub>2</sub> receptor expression. One crucial point concerning the action of BK is that the two receptors can be stimulated by the same agonist BK but with different affinities. Thus examination of the regulation of expression of BK receptors is a prerequisite in the understanding of BK action. At present, the regulation of the B<sub>2</sub> receptor in response to ligand binding or pathological factors has been examined in only a few studies (17, 19, 23, 54), but to our knowledge, no studies have been performed on the regulation of the B<sub>2</sub> receptor at the level of mRNA expression in renal tissue. Experiments on mesangial cells in culture have demonstrated that they respond to a number of vasoactive substances like AVP, angiotensin II, platelet-derived growth factor, adenosine, and endothelin by

contraction, whereas atrial natriuretic factor, dopamine, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), nitric oxide, guanosine 3',5'-cyclic monophosphate (cGMP), and adenosine 3',5'-cyclic monophosphate (cAMP) induce relaxation (for reviews, see Refs. 16, 42, 52). Because a number of these vasoactive factors can stimulate the formation of cAMP in mesangial cells and since a putative cAMP response element (CRE) exists in the 5'-flanking region of the B<sub>2</sub> receptor gene of the rat (46), the present study was designed to determine whether B<sub>2</sub> receptor expression and its related cellular responses are regulated by cAMP in rat mesangial cells. The intracellular cAMP concentration ([cAMP]<sub>i</sub>) was increased by treatment with forskolin or PGE<sub>2</sub>, and the possible functional changes of the B<sub>2</sub> receptor were assessed by measuring the BK-induced increase in free cytosolic calcium and prostaglandin secretion. The data show that elevating the intracellular level of cAMP increases both the expression of B<sub>2</sub> mRNA and the B<sub>2</sub> binding site density, accompanied by an increase in BK-induced prostaglandin secretion. However, no related changes in the BK-induced increase in free cytosolic calcium were observed.

## MATERIALS AND METHODS

**Materials.** Fetal calf serum (FCS), RPMI 1640, and collagenase were from Boehringer. Penicillin, streptomycin, glutamine, D-valine, BK, [D-Arg-Hyp<sup>3</sup>-D-Phe<sup>7</sup>]BK, cycloheximide, actinomycin D, forskolin, 3-isobutyl-1-methylxanthine (IBMX), 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrcAMP), PGE<sub>2</sub>, indomethacin, sucrose, phenanthroline, leupeptin, bacitracin, benzamidine, captopril, phosphoramidon, lysozyme, diethyl pyrocarbonate, and polyethylenimine were purchased from Sigma Chemical (Saint Quentin Fallavier, France). U-73122 was purchased from Calbiochem (France-Biochem), and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phalloidin (nitrobenzoxadiazole, NBD-phalloidin) and fura 2-acetoxymethyl ester (fura 2-AM) were from Molecular Probes (Interchim-Montluçon). cAMP was measured by enzyme immunoassay (EIA) (Cayman EIA kits; Cayman Chemical-SPI-BIO, Les Ullis, France). <sup>125</sup>I as sodium salt (400 mCi/ml) and [α-<sup>32</sup>P]dCTP (3,000 Ci/ml) were from ICN Pharmaceuticals. HOE-140 ([D-Arg-(Hyp<sup>3</sup>,Thi<sup>5</sup>,D-Tic<sup>7</sup>,Oic<sup>8</sup>)]BK) was a gift from P. B. Shöelkens (Hoechst, Frankfurt, Germany).

**Cell culture.** Rat glomerular mesangial cells were prepared and cultured as previously described (18). Briefly, primary mesangial cells were obtained as outgrowths of decapsulated collagenase-digested glomeruli, which were extracted under sterile conditions from the kidneys of 7-wk-old male Sprague-Dawley rats (Iffa Credo, Lyon, France). Glomerular explants were allowed to grow to confluence in RPMI 1640 medium (at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>) containing 15% fetal calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine, and D-valine substituted for L-valine to prevent fibroblast development (21). With this methodology, mesangial cells appeared in the culture after ~21–28 days. Mesangial cells were identified by morphological presence of multilayers, resistance to puromycin (10 µg/ml), sensitivity to mitomycin (5 µg/ml), presence of myosin filaments revealed by specific antibodies, presence of actin filaments revealed by fluorescent NBD-phalloidin, negative staining for factor VIII, and functional criteria (increase in intracellular calcium induced by angiotensin II), as previously described (18). Before treatment, confluent primary mesangial cells, ob-

tained after 21–28 days, were rendered quiescent by incubation in the same growth medium containing only 0.5% FCS (5). The FCS was necessary to maintain cell viability, as demonstrated by the trypan blue exclusion test in preliminary experiments. Based on previous results, we decided to use only primary mesangial cells to avoid variability due to cell passage.

**Binding studies.** Preparation of crude mesangial cell membranes and <sup>125</sup>I-labeled [Tyr<sup>0</sup>]BK was performed as described previously and used in binding studies, as currently performed in the laboratory (1, 18). Saturation studies were conducted at 37°C for 30 min in the presence of increasing amounts of <sup>125</sup>I-[Tyr<sup>0</sup>]BK (0.2–20 nM). The composition of the binding buffer was as follows: 5 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.4), 10 mM NaCl, 0.32 M sucrose, 2.5 mM phenanthroline, 10 µM leupeptin, 0.05% bacitracin, 1 mM benzamidine, 2 µM captopril, 1 µM phosphoramidon, and 0.1% lysozyme. The final assay volume was 0.4 ml. At the end of the 30-min incubation period, 4 ml of washing buffer (binding buffer without any inhibitor) was added, and the mixture was filtered on a GF/C Millipore filter (1.2 µm) that had been soaked prior to the assay in 0.1% polyethylenimine for 24 h, to reduce nonspecific binding. The filters were washed four times more with 4 ml of washing buffer, and the filter-bound radioactivity was determined in a gamma counter (Cristal multi-RIA, Packard). The specific binding was calculated as the difference between total and nonspecific binding obtained in the absence or presence of [Tyr<sup>0</sup>]BK (10 µM), respectively. Dissociation constant (K<sub>d</sub>) and maximum binding site density (B<sub>max</sub>) values were calculated from computer-assisted analysis of the data using the kinetic-EBDA-LIGAND program (Elsevier-Biosoft, Cambridge, UK) and expressed as femtomoles of iodinated BK bound per milligram of protein. For competition studies, fixed amounts of membrane extract were incubated for 30 min at 37°C in triplicate, with an increasing amount of unlabeled BK or antagonist (from 10<sup>-12</sup> to 10<sup>-4</sup> M) in the presence of either 0.5 nM or 2 nM of <sup>125</sup>I-[Tyr<sup>0</sup>]BK, according to the K<sub>d</sub> of the site 1 and site 2, respectively. Results are means ± SE of at least three independent assays carried out with different membrane pools. In each experiment, aliquots of cell culture were used to determine the protein content. Proteins were measured after solubilization for 15 min at 100°C with 1 M NaOH using the method of Lowry et al. (31), with bovine serum albumin as standard.

**Incubation protocols and assays for cAMP.** Cultured mesangial cells were seeded into six-well culture trays (Nunc) at a density of 5 × 10<sup>4</sup> cells/well and cultured with the complete medium for 48 h. Before treatment, cells were rendered quiescent by incubation for an additional 24-h period in the same growth medium containing 0.5% FCS. The cells were washed three times with 1 ml/well of phosphate-buffered saline before treatment for 6, 18, or 24 h with forskolin (0.1 µM) or PGE<sub>2</sub> (0.1 µM) in the presence of 0.5 mM IBMX. At the end of the incubation time, the reaction was stopped by removal of the medium, followed immediately by the addition of 1.6 ml of an ice-cold mixture of methanol and formic acid (95%/5%) to each well, so that only intracellular cAMP was measured. Measurements of intracellular cAMP were performed as previously described (4) using EIA kits (Cayman Chemicals).

**Measurements of D-myo-inositol 1,4,5-trisphosphate.** To measure D-myo-inositol 1,4,5-trisphosphate (IP<sub>3</sub>) production, mesangial cells were treated as described above. The production of IP<sub>3</sub> was assayed as previously described (1). Briefly, at the end of the incubation time (24 h) in the presence of either forskolin (0.1 µM), PGE<sub>2</sub> (0.1 µM), or 8-BrcAMP (100 µM), the culture medium was drawn off, replaced by fresh medium,



and stimulated for 30 s with BK (0.1  $\mu$ M). The stimulation was stopped by adding 10% perchloric acid followed by incubation on ice for 15 min. After neutralization with ice-cold 1.5 M KOH, the samples were centrifuged at 2,000 *g* for 15 min at 4°C. The supernatants were kept cold on ice, and aliquots of 100  $\mu$ l were used to measure the IP<sub>3</sub> concentration using the [<sup>3</sup>H]IP<sub>3</sub> RIA kit system (Amersham, Les Ullis, France).

**Measurements of intracellular calcium concentration.** The intracellular calcium concentration ( $[Ca^{2+}]_i$ ) was determined as currently done in the laboratory and previously described in detail (2, 4). After treatment with 8-BrcAMP (100  $\mu$ M), forskolin (0.1  $\mu$ M), or PGE<sub>2</sub> (0.1  $\mu$ M) in the presence of IBMX (0.5 mM) during the indicated time, mesangial cells were washed with Krebs-Ringer buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.4), 145 mM NaCl, 2.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 10 mM glucose, and 0.1% bovine serum albumin (BSA), loaded for 45 min in a 5  $\mu$ M solution of fura 2-AM at 37°C in the presence of 0.1% BSA, and washed twice before measuring the  $[Ca^{2+}]_i$  in response to BK (from 0.1 nM to 0.1  $\mu$ M) stimulation. Fluorescence measurements were done using a Spex Fluorilog spectrofluorometer, set for alternative dual-wavelength excitation at 340 and 380 nm. Light emitted at 505 nm was collected by a photomultiplier and passed to a Spex system microcomputer, which averaged the emission collected over a 0.5-s period at each excitation wavelength. Autofluorescence of unloaded cells was found to be ~18% of the emitted signal and was subtracted from the fura 2-loaded fluorescence at each excitation wavelength before calculating the fluorescence ratio R (340/380). As previously described,  $[Ca^{2+}]_i$  was calculated from the equation of Grynkiewicz et al. (22):  $[Ca^{2+}]_i = K_d \times (R - R_{min}/R_{max} - R) \times \lambda$ , where  $K_d$  (224 nM) is the dissociation constant of the complex fura 2-Ca<sup>2+</sup>, and  $R_{min}$ ,  $R_{max}$ , and  $\lambda$  are constant parameters depending on the optical system used. Under our experimental conditions, they were  $R_{min} = 0.8$ ,  $R_{max} = 16$ , and  $\lambda = 4$ .

**Induction of prostaglandin release and measurement.** Mesangial cells were treated as described above. At the end of the incubation time in the presence of either forskolin (0.1  $\mu$ M) or 8-BrcAMP (100  $\mu$ M), the culture medium was drawn off and replaced by fresh medium containing BK (0.1  $\mu$ M). The amount of prostaglandins secreted was determined after 5-min incubation at 37°C. After the 5-min incubation with BK, the supernatant was removed, centrifuged at 4,000 *g*, and the resulting supernatant was stored frozen until assayed for prostaglandins. The amount of PGE<sub>2</sub> secreted in the medium was assayed directly by specific EIAs (Cayman Chemical) (3). In brief, this assay is based on the competition between free prostaglandin and acetylcholinesterase-linked prostaglandin for rabbit prostaglandin antiserum. The prostaglandin antiserum was coated onto 96-well plates (Nunc certified) via a monoclonal anti-rabbit antibody. The enzymatic tracer (acetylcholinesterase) cleaves the Ellman reagent added to the well, and the colored substance released is inversely proportional to the amount of produced prostaglandin (expressed as picograms secreted during 5-min incubation per million of cells).

**RNA isolation and Northern blot analysis.** Mesangial cells were treated as described above, and total RNA was extracted as described previously (2). The RNA pellet was dried and dissolved in water containing diethyl pyrocarbonate (0.1%). RNA was quantified by ultraviolet spectrophotometry at 260 and 280 nm. Only RNA preparations with an OD<sub>260</sub>/OD<sub>280</sub> ratio between 1.9 and 2.1 were used for cDNA synthesis. RNA (25  $\mu$ g) was denatured and separated by electrophoresis on a 1.2% agarose/formaldehyde gel and transferred overnight

onto a standard nylon membrane (Hybond-N; Amersham, Les Ullis, France) with 20 $\times$  SSC (standard sodium citrate) as the transfer buffer. The RNA was fixed to the membrane under ultraviolet radiation (254 nm) in a ultraviolet crosslinker (Stratagene). Membrane filters were prehybridized for at least 4 h at 42°C in a phosphate buffer (pH 6.5) containing 45% deionized formamide, 4 $\times$  SSC, 5 $\times$  Denhardt's reagent (Sigma), 0.1% sodium dodecyl sulfate (SDS), and 7.5  $\mu$ g/ml of denaturated salmon sperm (Sigma). Hybridization was performed overnight at 42°C using hybridization buffer (0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 6.5, 45% deionized formamide, 4 $\times$  SSC, 1 $\times$  Denhardt's reagent, 0.1% SDS, and 7.5  $\mu$ g/ml of denaturated salmon sperm) and, as a probe, a 1.4-kb fragment corresponding to the coding region of the rat B<sub>2</sub> receptor (46), labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by random priming (Amersham Megaprime Kit). The membrane was washed once with 2 $\times$  SSC/0.1% SDS at room temperature and twice at 65°C for 30 min. Autoradiography was performed for 1–4 days at –80°C, using Amersham Hyperfilm-MP film with intensifying screens.

After hybridization with the B<sub>2</sub> receptor probe and exposure, blots were washed and rehybridized with a  $\beta$ -actin cDNA probe, obtained by polymerase chain reaction, as previously described (2), to control for loading and transfer of total RNA in individual samples. The quantity of B<sub>2</sub> receptor and  $\beta$ -actin mRNAs was evaluated by scanning the membrane with a PhosphorImager (PhosphorImager 445, Molecular Dynamics) with which the densitometric value of each band was calculated. The B<sub>2</sub> receptor mRNA expression was normalized to the densitometric values obtained for the constitutively expressed  $\beta$ -actin mRNA signal.

**Statistical analysis.** Values are expressed as means  $\pm$  SE of 3–6 independent experiments. The nonparametric Mann-Whitney *U*-test was used for comparisons between two unpaired variables. Multiple means were compared using single-factor analysis of variance. Differences were considered significant at  $P < 0.01$  or  $P < 0.05$ .

## RESULTS

**cAMP production in forskolin and PGE<sub>2</sub>-treated mesangial cells.** The effect of forskolin (0.1  $\mu$ M) and PGE<sub>2</sub> (0.1  $\mu$ M) treatment in the presence of an inhibitor of phosphodiesterase (IBMX, 0.5 mM) for 6, 18, and 24 h on the mesangial cell  $[cAMP]_i$  content is shown in Fig. 1. Both forskolin and PGE<sub>2</sub> treatment increased significantly the  $[cAMP]_i$  by three- and twofold, respec-

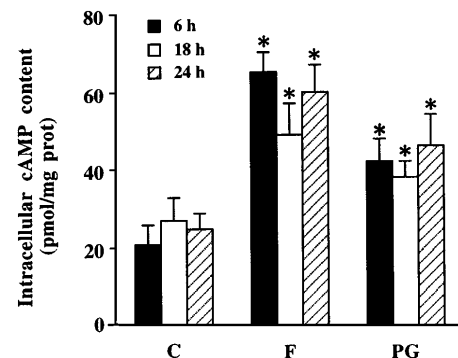


Fig. 1. Effect of forskolin (F, 0.1  $\mu$ M) and prostaglandin E<sub>2</sub> [PGE<sub>2</sub> (PG), 0.1  $\mu$ M] treatment in the presence of 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM) for 6, 18, and 24 h on the mesangial cell cAMP content. C, control; prot, protein. Data are representative of 4 independent experiments. \* $P < 0.05$  compared with respective control.

tively, compared with untreated control cells. For the different treatment times, there was no significant difference in the amount of cAMP formed. This is consistent with previously reported data (4, 10). However, an equimolar forskolin concentration was more potent than PGE<sub>2</sub> to increase [cAMP]<sub>i</sub>. Furthermore, it has been reported that cAMP can induce morphological changes and can affect mesangial cell adhesion (27). Therefore, we counted the cell number, as described previously (5), before and after the treatment with either forskolin or PGE<sub>2</sub> in presence of IBMX. At the concentrations of these agents that increase the mesangial cell [cAMP]<sub>i</sub> used in this study, no variation in the cell number was observed (data not shown).

**Effect of [cAMP]<sub>i</sub>-stimulating agents and 8-BrcAMP on the density of B<sub>2</sub> binding sites.** The presence of B<sub>2</sub> receptors on mesangial cells (5) was verified in binding studies using <sup>125</sup>I-[Tyr<sup>0</sup>]BK. The specificity of the binding was that of a typical B<sub>2</sub> receptor binding site, since the specific B<sub>1</sub> antagonist, des-Arg<sup>9</sup>-Leu<sup>8</sup>-BK, was unable to displace <sup>125</sup>I-[Tyr<sup>0</sup>]BK in competition studies, where the relative order of potency in displacing <sup>125</sup>I-[Tyr<sup>0</sup>]BK binding was HOE-140 > BK > [D(Arg)-Hyp<sup>3</sup>-D-Phe<sup>7</sup>]BK (data not shown). Despite a significant increase in the [cAMP]<sub>i</sub> after 6 h of treatment with the different cAMP-stimulating agents (Fig. 1), there was a delay before any detectable increase in the B<sub>2</sub> receptor binding site density could be observed (Fig. 2A). As previously described (5), in mesangial cells, the population of binding sites included a small number (B<sub>max</sub> close to 20 fmol/mg protein) of high-affinity sites (K<sub>d</sub> < 1 nM) and a large number (B<sub>max</sub> > 80 fmol/mg protein) with an affinity ~3 nM (Table 1). Whereas the density of the high-affinity BK binding sites was not affected (Table 1) by the chronic treatment with forskolin (0.1 μM), PGE<sub>2</sub> (0.1 μM), and 8-BrcAMP (100 μM), the density of the low-affinity population of BK binding sites was significantly increased after 24 h of treatment (Fig. 2A). After 24-h treatment, B<sub>max</sub> values were 89 ± 9, 148 ± 11, 125 ± 10, and 131 ± 7 fmol/mg protein for the control, forskolin, PGE<sub>2</sub>, and 8-BrcAMP-treated mesangial cells, respectively. The receptor affinity was unchanged and remained in the nanomolar range (Table 1). Moreover, the effect of 24-h treatment with [cAMP]<sub>i</sub> elevating agents on the density of the low-affinity BK binding sites was dose dependent, as shown in Fig. 2B. Furthermore, the increase in B<sub>2</sub> receptor binding site density induced by 24-h treatment with forskolin, PGE<sub>2</sub>, or 8-BrcAMP was completely inhibited with the protein synthesis inhibitor cycloheximide (5 μg/ml, Fig. 2C).

**Effect of [cAMP]<sub>i</sub>-stimulating agents on B<sub>2</sub> receptor mRNA expression.** Northern blot analysis of total RNA from primary cultured rat mesangial cells using a B<sub>2</sub> receptor probe demonstrated the presence of a 4.2-kb band, which is in agreement with the expected size for B<sub>2</sub> receptor mRNA (Fig. 3A). When the results were normalized by calculating the ratio B<sub>2</sub>/β-actin, a significant increase in B<sub>2</sub> receptor mRNA was observed in forskolin-treated cells for 18 and 24 h and PGE<sub>2</sub>-treated cells for 24 h (Fig. 3B). The strongest increase was

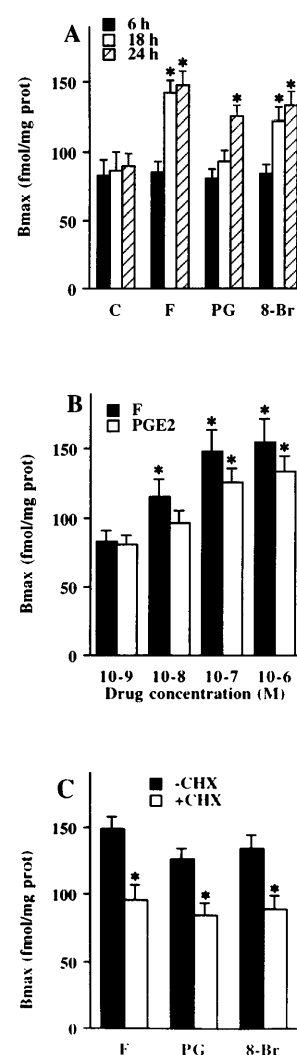


Fig. 2. *A*: effect of forskolin (0.1 μM), PGE<sub>2</sub> (0.1 μM), and 8-BrcAMP (8-Br, 100 μM) treatment for 6, 18, and 24 h on the bradykinin B<sub>2</sub> receptor density on mesangial cells. *B*: dose effect of forskolin and PGE<sub>2</sub> treatment (24 h) on B<sub>2</sub> receptor density. *C*: effect of coincubation, for 24 h, of forskolin (0.1 μM), PGE<sub>2</sub> (0.1 μM), or 8-BrcAMP (100 μM) with (+CHX) or without (–CHX) the protein synthesis inhibitor cycloheximide (CHX, 5 μg/ml). B<sub>max</sub>, maximum binding site density. Data are representative of 4 independent experiments. \* *P* < 0.01 compared with the respective control.

observed in forskolin-treated cells for 24 h, where the intensity of the 4.2-kb B<sub>2</sub> receptor message increased by 160% compared with the control value (100%) at the same time. As shown in Fig. 4, the effect of 24-h treatment with cAMP elevating agents was dose related. Because the effect of increasing the [cAMP]<sub>i</sub> suggested a de novo synthesis of B<sub>2</sub> receptors, the experiments were repeated in the presence of the transcriptional inhibitor actinomycin D (5 μg/ml) for 24 h. As shown in Table 1, actinomycin D inhibited the increase in the number of low-affinity BK binding sites induced by the different agents. Whereas actinomycin D was without significant effect on the control cells (not shown), it prevented the increase in B<sub>2</sub> receptor mRNA expression (Fig. 5).

**Effect of 8-BrcAMP and [cAMP]<sub>i</sub>-stimulating agents on BK-stimulated IP<sub>3</sub> formation.** We have reported that in nontreated mesangial cells, B<sub>2</sub> receptor activation by

Table 1. Effect of forskolin (0.1  $\mu$ M), PGE<sub>2</sub> (0.1  $\mu$ M), and 8-BrcAMP (100  $\mu$ M) treatment (24 h) in the absence (-) or presence (+) of actinomycin D (5  $\mu$ g/ml) on the bradykinin B<sub>2</sub> receptor density on mesangial cells

	- Actinomycin D				+ Actinomycin D	
	site 1		site 2		site 2	
	B <sub>max</sub>	K <sub>d</sub>	B <sub>max</sub>	K <sub>d</sub>	B <sub>max</sub>	K <sub>d</sub>
Control	13.4 ± 2	0.4 ± 0.1	89 ± 9	3.2 ± 1.3	92 ± 13	3.4 ± 1.2
Forskolin	15.7 ± 3	0.5 ± 0.2	148 ± 11*	3.6 ± 1.1	101 ± 9†	3.1 ± 1.0
PGE <sub>2</sub>	11.2 ± 2	0.35 ± 0.1	125 ± 10*	2.9 ± 0.9	87 ± 8†	2.6 ± 0.8
8-BrcAMP	14 ± 4	0.44 ± 0.2	131 ± 7*	3.3 ± 0.8	90 ± 6†	2.8 ± 1.3

Values are means ± SE of 3 independent experiments, where each point was run in triplicate. Maximum binding site density (B<sub>max</sub>) is expressed in fmol/mg protein, and dissociation constant (K<sub>d</sub>) is in nM. Site 1 is the high-affinity bradykinin binding site, and site 2 is the low-affinity bradykinin binding site. PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; 8-BrcAMP, 8-bromoadenosine 3',5'-cyclic monophosphate. \*  $P < 0.01$  compared with control in the absence of actinomycin D. †  $P < 0.01$  compared with similar assay in the absence of actinomycin D.

BK induces a rapid and transient release of IP<sub>3</sub> with a peak value after 30 s of BK stimulation (1). To verify whether the cAMP-induced increase of B<sub>2</sub> receptor number had any effect on BK-induced conversion of phosphatidylinositol 4,5-bisphosphate by PLC to IP<sub>3</sub>, we measured IP<sub>3</sub> production after 24 h of forskolin (0.1  $\mu$ M), PGE<sub>2</sub> (0.1  $\mu$ M), and 8-BrcAMP (100  $\mu$ M) treatment. No clear differences were observed. In control cells, IP<sub>3</sub> production increased from 52 ± 5 to 165 ± 13 pmol/mg protein on stimulation with BK (0.1  $\mu$ M). After treatment with forskolin, PGE<sub>2</sub>, or 8-BrcAMP, IP<sub>3</sub> production increased from 49 ± 7 to 176 ± 16 pmol/mg protein on BK stimulation (0.1  $\mu$ M).

Effect of 8-BrcAMP and [cAMP]<sub>i</sub>-stimulating agents on the BK-induced intracellular calcium increase. As

shown in Fig. 6A, addition of BK (0.1  $\mu$ M) to adherent mesangial cells induced a rapid and transient rise in [Ca<sup>2+</sup>]<sub>i</sub>, reaching a peak value which then declined to a stable resting level (plateau). Treatment with either forskolin (0.1  $\mu$ M), PGE<sub>2</sub> (0.1  $\mu$ M), or 8-BrcAMP (100  $\mu$ M) for 6, 18, or 24 h did not significantly modify either the basal free cytosolic calcium or the mobilization (transient and sustained phases) of [Ca<sup>2+</sup>]<sub>i</sub> induced by 0.1  $\mu$ M BK (Table 2, Fig. 6B). As previously described (5), the effect of BK on the [Ca<sup>2+</sup>]<sub>i</sub> was dose dependent (Table 2). Neither the basal [Ca<sup>2+</sup>]<sub>i</sub> nor the dose-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> in response to BK in the range from 0.1 nM to 0.1  $\mu$ M was affected by the treatment with forskolin, PGE<sub>2</sub>, or 8-BrcAMP (Table 2).

Effect of 24 h forskolin and 8-BrcAMP treatment on BK-induced prostaglandin release. Because activation of the B<sub>2</sub> receptor by BK is also known to stimulate the PLA<sub>2</sub> pathway (9), we have examined the effect of BK on the PGE<sub>2</sub> production. In untreated mesangial cells, BK (0.1  $\mu$ M) increased the basal value from 285 ± 35 to 2,842 ± 230 pg PGE<sub>2</sub> · 5 min<sup>-1</sup> · 1 million cells<sup>-1</sup> (Fig. 7). The BK stimulation of cells pretreated with either forskolin (0.1  $\mu$ M) or 8-BrcAMP (100  $\mu$ M) for 24 h induced a further increase of, respectively, 4,253 ± 310 and 4,052 ± 286 pg PGE<sub>2</sub> · 5 min<sup>-1</sup> · 1 million cells<sup>-1</sup>. The increase in BK-induced PGE<sub>2</sub>-secretion observed

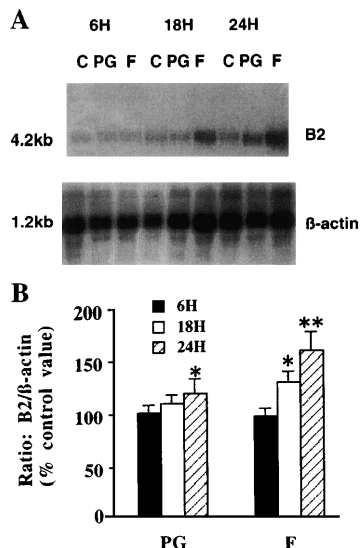


Fig. 3. Effects of an increased intracellular cAMP concentration on B<sub>2</sub> receptor mRNA expression. A: representative autoradiogram of a Northern blot analysis of effects of forskolin (0.1  $\mu$ M) and PGE<sub>2</sub> (0.1  $\mu$ M) treatment for 6, 18, and 24 h on B<sub>2</sub> receptor mRNA expression in cultured rat mesangial cells. Total RNA was extracted after different times of exposure to the drug indicated, followed by analysis of 25  $\mu$ g of RNA with a B<sub>2</sub> receptor probe (B<sub>2</sub>), as described in MATERIALS AND METHODS. After dehybridization, the same filter was rehybridized with a  $\beta$ -actin probe ( $\beta$ -actin). Data shown are representative of 4 experiments. B: ratio of densitometric values of B<sub>2</sub> receptor to  $\beta$ -actin. Each value is the mean of 4 independent experiments and is expressed in % of control value (100%) for each incubation time. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

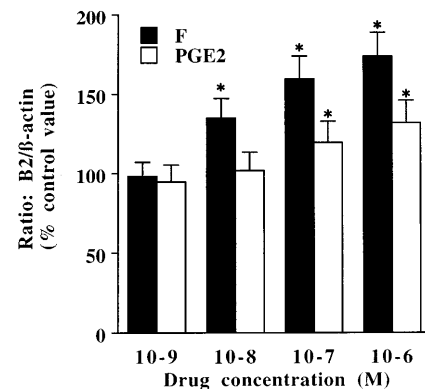


Fig. 4. Dose effect of forskolin and PGE<sub>2</sub> treatment (24 h) on the expression of B<sub>2</sub> receptor mRNA in cultured rat mesangial cells. Data are representative of 3 independent experiments. Data show ratios of densitometric values of B<sub>2</sub> receptor to  $\beta$ -actin. Each is expressed in % of control value (100%) for each incubation time. \*  $P < 0.05$ , compared with respective control.



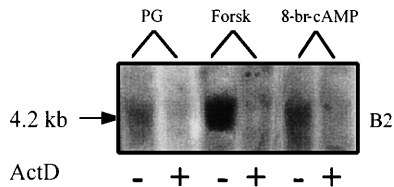


Fig. 5. Representative autoradiogram of a Northern blot analysis (25  $\mu$ g total RNA) of effect of forskolin (Forsk, 0.1  $\mu$ M), PGE<sub>2</sub> (0.1  $\mu$ M), and 8-BrcAMP (100  $\mu$ M) treatment for 24 h in absence (-) or presence (+) of actinomycin D (ActD, 5  $\mu$ g/ml) on expression of B<sub>2</sub> receptor mRNA in cultured rat mesangial cells. Data are representative of 3 experiments.

in cells treated with forskolin or 8-BrcAMP was abolished by indomethacin (1  $\mu$ M) but not by the PLC inhibitor U-73122 (0.5  $\mu$ M) added during the 5-min incubation period with BK (not shown).

## DISCUSSION

In the present study, we focused our attention to the relationship between the cAMP-induced stimulation of the expression of the B<sub>2</sub> receptor and its related cellular responses. We have demonstrated that, in primary culture of rat mesangial cells, agents increasing the intracellular cAMP levels also increase B<sub>2</sub> receptor mRNA levels, as well as the B<sub>2</sub> receptor density. Furthermore, actinomycin D, a transcriptional inhibitor, and cycloheximide, a protein synthesis inhibitor, completely inhibited the cAMP-induced rises in B<sub>2</sub> mRNA and B<sub>2</sub> receptor binding. We have also examined whether this increase in B<sub>2</sub> receptor density modified its related transduction pathways. Whereas the increase in mRNA level and binding sites were accompa-

Table 2. Effect of 24 h of pretreatment with forskolin (0.1  $\mu$ M), PGE<sub>2</sub> (0.1  $\mu$ M), and 8-BrcAMP (100  $\mu$ M) on the intracellular calcium concentration increase induced by 0.1 nM, 1 nM, 10 nM, and 0.1  $\mu$ M BK

BK Concentration	Basal Level	0.1 nM	1 nM	10 nM	0.1 $\mu$ M
Control	92 $\pm$ 12	160 $\pm$ 12	220 $\pm$ 18	275 $\pm$ 29	342 $\pm$ 42
Forskolin	87 $\pm$ 11	165 $\pm$ 9	230 $\pm$ 22	280 $\pm$ 35	347 $\pm$ 51
PGE <sub>2</sub>	90 $\pm$ 13	162 $\pm$ 15	224 $\pm$ 20	274 $\pm$ 31	343 $\pm$ 45
8-BrcAMP	85 $\pm$ 14	159 $\pm$ 13	219 $\pm$ 20	278 $\pm$ 28	344 $\pm$ 44

Values are means  $\pm$  SE. Each value represents the mean peak value of 6 separate coverslips and is expressed in nM calcium. When compared with respective basal level value, all calcium concentration values are significantly different ( $P < 0.01$ ).

nied by an increase in the BK-stimulated release of PGE<sub>2</sub>, no effect on BK-induced IP<sub>3</sub> production and calcium second messenger production was observed. Therefore, these data bring new information regarding the dual signaling pathways of B<sub>2</sub> receptors observed in various cell types.

The effect of cAMP on BK receptor density and on BK-induced signal transduction has been investigated (14, 15, 20, 36), and conflicting results were reported depending on the cell type studied and on the duration of treatment with agents that increase the intracellular cAMP concentration. In human fibroblasts, Etscheid et al. (20) have reported that cholera toxin, pertussis toxin, and forskolin induced a fourfold increase in the number of BK receptors associated with an enhanced arachidonic acid release in response to BK, which is in good agreement with our results. In human tracheal epithelial cells (14), very short-term pretreatment (5 min) with isoproterenol, which is known to increase the cAMP concentration, did not affect the calcium response to BK, indicating only that cAMP did not interfere with the BK-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> in this cell type. In canine tracheal smooth muscle cells (TSMC), long-term (24 h), but not short-term (<4 h), treatment with agents that increase intracellular cAMP enhanced both BK receptor binding and BK-induced increases in inositol phosphates and intracellular cal-

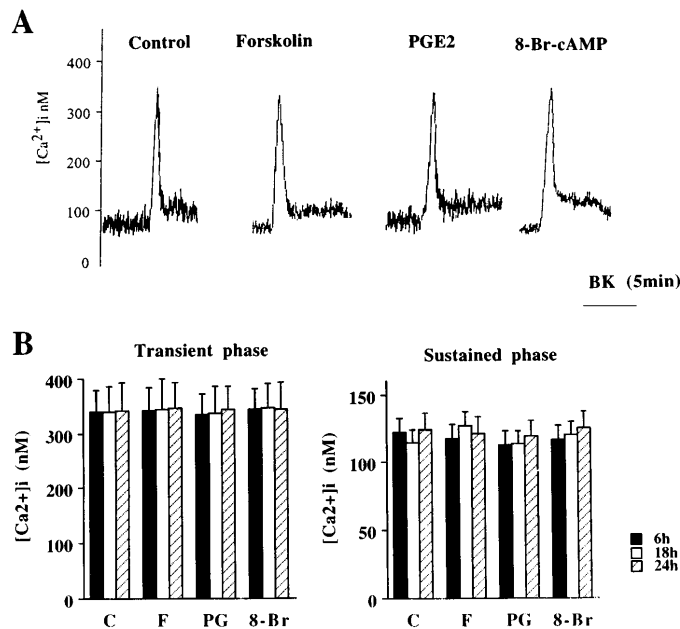


Fig. 6. Effect of forskolin (0.1  $\mu$ M), PGE<sub>2</sub> (0.1  $\mu$ M), and 8-BrcAMP (100  $\mu$ M) treatment, for 6, 18, and 24 h on stimulation of intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>) induced by bradykinin (BK, 0.1  $\mu$ M) on adherent mesangial cells. *A*: representative profiles (obtained after 24 h of treatment). *B*: mean peak values. Each value is the mean of 6 separate coverslips.

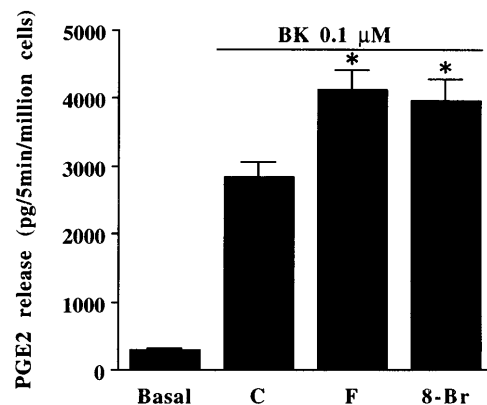


Fig. 7. Effect of 24 h treatment with forskolin (0.1  $\mu$ M) and 8-BrcAMP (100  $\mu$ M) on PGE<sub>2</sub> production induced by BK (0.1  $\mu$ M) on rat mesangial cells. Basal indicates production of PGE<sub>2</sub> without addition of BK. C, F, and 8-Br cells were with BK. Data are expressed as means  $\pm$  SE of 3 independent experiments. \*  $P < 0.01$  compared with respective control.



cium (36). However, Mao Yang et al. (36) used a 100-fold higher dose of forskolin than the one we determined as having the maximum cAMP-releasing capacity. Another likely explanation is that the control TSMC partially lost their BK receptors or have a much lower receptor density than in mesangial cells. Although pretreatment with 10  $\mu$ M forskolin for 24 h in these studies (36) induced an eightfold increase in BK-binding sites, the stimulated  $[Ca^{2+}]_i$  level was similar to the level we have observed in mesangial cells before challenging the cAMP production. A similar increase in B<sub>2</sub> receptor synthesis was evoked, by increasing the  $[cAMP]_i$  in rat arterial smooth muscle cells (15). However, the increase in B<sub>2</sub> receptor synthesis was determined using binding analysis, and the receptor mRNA level was not examined. Moreover, in this study, the author shows that only long-term stimulation with cAMP (24 h) enhanced both BK binding sites and BK-stimulated calcium mobilization, whereas short-term stimulation with cAMP produced a variable inhibition of BK-stimulated calcium mobilization, depending on the passage number of the cells (15). In these studies, a 200-fold higher dose of forskolin (20  $\mu$ M) was used. Finally, in contrast to previous work on mesangial cells (44), including our present data, Dixon (15) observed a very small effect of PGE<sub>2</sub> treatment on the increase of  $[cAMP]_i$  without any effect on the number of BK receptor binding sites (15).

Most of the studies, except those of Etscheid et al. (20), describe only the changes in the cellular responses of BK by its effect on  $[Ca^{2+}]_i$  mobilization. Indeed, it has been hypothesized for a long time that BK mediated all its cellular responses through PLC activation and that stimulation of PLA<sub>2</sub> was considered as a PLC-dependent mechanism. The lack of increase in  $[Ca^{2+}]_i$  following chronic cAMP treatment seems to be specific for the BK transduction pathway, since we observed a decrease in  $[Ca^{2+}]_i$  mobilization in response to angiotensin II (0.1  $\mu$ M) after 24 h treatment with forskolin (data not shown). Such a phenomenon was also observed in rat arterial smooth muscle cells (15). Interestingly, our data show that only the low-affinity population of BK receptors was increased by agents that increase the intracellular cAMP level and that this was accompanied by an enhanced release of PGE<sub>2</sub> induced by BK, suggesting that the newly synthesized receptors are principally coupled to the PLA<sub>2</sub> signaling pathway. This is supported by the observation that BK-induced PGE<sub>2</sub> production was inhibited by PLA<sub>2</sub> inhibitor indomethacin but not by PLC inhibition by U-73122. These data are consistent with previous studies showing that BK-induced activation of PLC and PLA<sub>2</sub> are partly independent (9, 25). In addition, we have previously demonstrated that BK induced contraction via two independent mechanisms, one associated with the PLC pathway and one dependent on prostaglandin formation (3).

This selective coupling of the newly synthesized, low-affinity B<sub>2</sub> receptors might be explained by the existence of multiple-affinity states of G protein-coupled receptors (8, 11, 51). These multiple-affinity

states are thought to be regulated by binding of different G proteins (8, 11, 51) and/or by phosphorylation of Ser/Thr residues on the receptor occurring after activation of the human B<sub>2</sub> receptor (7). Furthermore the B<sub>2</sub> receptor was shown to couple at least to three different G proteins (29, 30). In this context, it is interesting to note that there is clearly one B<sub>2</sub> receptor mRNA (Refs. 24, 39, 40, this work), but several groups have reported two BK binding sites (28, 34, 45).

cAMP is involved in the up- or downregulation of receptor expression of some vasoactive agents, such as angiotensin II (12, 13, 33) and endothelin (43), in various cell types. A well-known mechanism, whereby cAMP regulates gene transcription, involves the phosphorylation by protein kinase A and subsequent activation of transcription factor CREB, the CRE binding protein, which binds to the CRE located in the 5'-untranslated region of these genes (50). We found that increased intracellular concentration of cAMP induced B<sub>2</sub> receptor mRNA expression. This result is consistent with the presence of a CRE located in the promoter region of the B<sub>2</sub> receptor gene in rat (46). Indeed, a recent study of Pesquero et al. (47) showed that part of the B<sub>2</sub> receptor promoter region containing the CRE (a 1-kb fragment) induced reporter gene activity on treatment with the cAMP analog, 8-BrcAMP. Although these and our data do not exclude the possibility of a stabilizing effect of cAMP on B<sub>2</sub>-mRNA, they favor induction of transcription by cAMP.

It is now well admitted that glomerular mesangial cells increase their intracellular cAMP concentration in response to a wide spectrum of humoral agents like parathyroid hormone, arginine vasopressin, serotonin, histamine, prostaglandins, and isoproterenol (for reviews, see Refs. 16, 42). Glomerular diseases are associated with mesangial cell dysfunctions, such as alteration in cell contraction, cell proliferation, and matrix secretion (26), where cyclic nucleotides play an important role. Therefore, the physiological significance of the present results should be related to pathological states like glomerular inflammation. In these situations, mesangial cells are both producers of and targets for a variety of cytokines, eicosanoids, and reactive oxygen species. All these inflammatory agents mediate part of their cellular effects by stimulating cAMP and cGMP production. Furthermore, it has been shown that, in glomeruli, accumulation of cyclic nucleotides is higher than in renal tubules (53). Moreover, cAMP and cGMP accumulation can also result from a decreased activity of their degradation pathway involving phosphodiesterase (PDE). Although PDE activity has not been extensively investigated in renal diseases, a decrease in PDE activity was recently reported in rat with unilateral obstruction (38). In this respect, the increase in functional B<sub>2</sub> receptor by an increased cAMP concentration only associated with the increase in PGE<sub>2</sub> production is of potential interest. Indeed, although the major described function of PGE<sub>2</sub> in the glomerulus is the maintenance of glomerular filtration by counteracting the effects of vasoconstrictive peptides (42), other important effects have been assigned to PGE<sub>2</sub>. It has been

demonstrated that PGE<sub>2</sub> inhibits growth of mesangial cells via the inhibition of MAP kinase (55) and that PGE<sub>2</sub> reduces the expression and secretion of collagen (56).

In conclusion, we provide new evidence that expression of a G protein-coupled receptor can be upregulated by cAMP not only at the level of the receptor expression but also at the level of the second messenger pathway. Finally, the selective stimulating effect of cAMP on BK-induced PGE<sub>2</sub> secretion through stimulation of B<sub>2</sub> receptor expression provides strong evidence for the existence of a dual and independent signaling pathway of this receptor, although the coupling mechanism remains to be investigated.

We thank Dr. P. Winterton for revising the English version.

This work was partly funded by grant from région Midi-Pyrénées (RECH/9407562).

M. E. Marin Castaño is supported by a grant from the French Nephrology Society. J. P. Schanstra is recipient of a postdoctoral fellowship position from Institut National de la Santé et de la Recherche Médicale.

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Received 9 May 1997; accepted in final form 21 November 1997.

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