Bradykinin stimulates production of inositol (1,4,5) trisphosphate in cultured mesangial cells of the rat via a BK₂-kinin receptor

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1 Using [^{125}I-Tyr^0]-BK, as radiolabelled ligand, and various agonists and antagonists of bradykinin (BK) we identified a single class of specific BK₂-binding sites in mesangial cell membranes (B_{max} = 73 fmol mg^{-1} protein and K_d = 3.7 nM).

2 Following the addition of 0.1 μM BK, inositol (1,4,5) trisphosphate (IP3) formation increased within 20 s from a basal level of 64 to a maximal value of 175 pmol mg^{-1} protein.

3 Incubation in a Ca^{2+}-free medium did not change IP3 production but a 5 min preincubation with 1 mM EGTA completely prevented the BK-induced IP3 formation, suggesting that IP3 formation is partly dependent on extracellular calcium.

4 The BK₂ antagonist D-Arg-Hyp^3-D-Phe^7-BK (10 μM) but not the BK₁ antagonist (des-Arg^9-Leu^8-BK) abolished IP3 production in response to 0.1 μM BK. Pretreatment of mesangial cells with pertussis toxin was without effect on BK-induced IP3 formation, whereas phorbol 12-myristate 13-acetate significantly enhanced (by 25%) BK-induced IP3 formation.

5 The present data demonstrate that inositol phosphate breakdown in rat mesangial cells can be mediated via activation of a BK₂-kinin receptor and is under negative control of protein-kinase C.

Keywords: Bradykinin; rat cultured renal mesangial cells; inositol (1,4,5) trisphosphate; BK₂-receptor

Introduction

Mesangial cells, which represent about one third of the glomerular cell population, are contractile cells of the renal glomerulus (Kreisberg et al., 1985; Schlondorff, 1987) that respond to vasoactive stimuli. It has been hypothesized that both contraction and relaxation of mesangial cells are involved in the regulation of the glomerular filtration rate. Contraction and relaxation of mesangial cells have been observed in response to a large number of agents (Pfeilschifter, 1989; Mene et al., 1989). Agents such as angiotensin II, vasopressin, noradrenaline, platelet activating factor and thrombin, induce cell contraction; atriopeptide, guanosine 3':5'-cyclic monophosphate (cyclic GMP), cyclic AMP, prostaglandin E₂ (PGE₂) and dopamine are relaxing factors. Among the vasoactive agents, the effect of the nonapeptide bradykinin (BK) on mesangial cells has received less attention. However, micropuncture studies pointed out that intraglomerularly infused BK decreased the glomerular ultra-filtration coefficient (Kf) in rat (Baylis et al., 1976), but at that time no mechanism was proposed. Kf is the product of the transcapillary hydraulic permeability and the total filtration area which is directly related to the contractile state of the cells. More recently it was demonstrated that BK stimulates a rise in cytosolic calcium in glomerular mesangial cells (Kremer et al., 1987). Whether this rise in intracellular free calcium concentration is due to influx from extracellular calcium space or to mobilization of the intracellular store has not yet been fully investigated. On the basis of these preliminary observations, we recently characterized the presence of BK₂ receptor-like binding in glomerular membrane (Bascands et al., 1989). The density of this binding site appeared to be down-regulated by the level of activity of the kinin-forming enzyme during changes in sodium intake (Emond et al., 1989).

To confirm further two recent reports from our laboratory we investigate here the early response of cultured mesangial cells to BK and quantify this response by measuring inositol (1,4,5) trisphosphate (IP3) formation.

Methods

Cell culture

Mesangial cells were obtained following culture of glomeruli isolated by graded sieving as described by Foidart et al. (1979). Under sterile conditions the kidneys were excised from anaesthetized normal Sprague Dawley rats (body weight 140–200 g), the cortex was removed, minced and washed several times in Hank's balanced salt solution and treated with collagenase (0.35 u l^{-1}). The tissue suspensions were then passed through three consecutive sterilized filters with decreasing pore sizes: 180, 125 and 75 μm. The isolated glomeruli were harvested from the surface of the 75 μm filter and plated into 25 cm² flask (NUNC) in a humidified atmosphere of 95% air 5% CO₂ and grown in complete medium consisting of RPMI 1640 supplemented with 15% foetal calf serum, 50 u ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 2 mM glutamine. Mesangial cells can be distinguished from fibroblasts by their ability to grow in a D-valine-containing medium (Gilbert & Migeon, 1975). In these conditions, it is well established that the first cell type to grow is the epithelial cells which persist for about one week and then start to senesce while with the high concentration of foetal calf serum used, the mesangial cells appear rapidly and proceed to overgrow the epithelial cells after 3 weeks of culture (Foidart et al., 1979). The mesangial cells were identified by morphological and functional criteria: under phase-contrast microscopy, cells appeared large and stellate. The cells are sensitive to mitomycin (5 μg ml^{-1}) and do not stain with factor VIII antigen. Mesangial cells showed histochemical evidence of actin fibres revealed by NBD fluorescent phallacidin and myosin filaments revealed by specific antibodies. Angiotensin II (10^{-8} M)

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and vasopressin (10^{-9} M) induced contraction of the cells and PGE$_2$ (10^{-8} M) relaxed the cells. As epithelial cells do not have angiotensin II receptors, the cell population obtained after 3 weeks of culture is a homogeneous mesangial cell culture, not contaminated with epithelial cells.

Membrane preparation and binding studies

The cultured cells were scraped off and suspended in the following medium: 50 mM Tris-HCl pH 7.4 containing 5 mM EDTA, 100 mM benzamidine, 10 mM leupeptin, 1 mM NaHCO$_3$, 2.5 mM MgCl$_2$, and manually homogenized in a tissue grinder (KONTES). The homogenate was centrifuged at 40,000 g for 30 min and the pellet was used as membrane preparation. Proteins were measured by the method of Lowry et al. (1951) after solubilization for 15 min at 100°C with 1 M NaOH; repeated cell counts revealed that 200 ± 50 µg of cellular protein was equivalent to 10^6 cells. The radiolabelled bradykinin used was [^{125}I-Tyr^0]-BK iodinated with Na^{125}I (Amersham) in the laboratory by the chloramin T method and immediately purified by high performance liquid chromatography (h.p.l.c.); the specific radioactivity was 280 Ci mmol^{-1}. Binding experiments were conducted as recently described (Bascands et al., 1989). Briefly, saturation studies were performed with amounts of [^{125}I-Tyr^0]-BK increasing from 1 to 10 nM. For inhibition studies, a fixed concentration of [^{125}I-Tyr^0]-BK (1.7 nM) was used in the presence of increasing concentrations of agonists or antagonists (from 10^{-11} to 10^{-4} M) but also with other peptides for which receptors have been identified in mesangial cells such as angiotensin II, vasopressin and atrial natriuretic factor; the residual binding was expressed as the percentage of total specific binding. In both types of study, the final volume was 0.4 ml, the binding buffer consisted of 5 mM potassium phosphate, pH 7.2, containing 0.32 M sucrose and 0.1% lysozyme. After a 45 min incubation time at 37°C, 4 ml of washing buffer (binding buffer without lysozyme) was added and the total volume filtered on a GF/C Whatman filter previously soaked for at least 2 h in polyethylenimine (1%). The filters were washed four additional times with 4 ml of washing buffer. The filter-bound radioactivity was determined in a Cristal Multi RIA Packard gamma-counter. Specific binding was calculated by subtracting binding in the presence of excess unlabelled BK (10 µM) from the total binding in the absence of unlabelled peptide. The results of the saturation studies, the binding parameters (B_{max}, K_d), were analysed by use of the Kinetic EBDA Ligand computerized programme (Munson & Rodbard, 1980) which gives the linear transformation of the saturation data (Scatchard analysis, Hill plot (nH)). B_{max} is expressed as fmol of bradykinin bound per mg of protein (fmol mg^{-1} prot.) and K_d in nM. For competition studies the relative affinities of the different competitors were determined with one- or two-site models by the computerized programme mentioned above. Values are means ± s.e. of triplicate measurements of three independent experiments.

D-myo-Inositol (1,4,5) phosphate production and assay

After 21 days of culture, mesangial cells were seeded in 6-well culture trays (NUNC) at a density of 10^5 cells/well and cultured for 72 h with the complete medium. The cells were rendered quiescent by incubation in serum-free buffered medium for 24 h. After the 24 h period, some cultures were pretreated for 12 h with phorbol 12-myristate 13-acetate (PMA) 50 ng ml^{-1} or for 4 h with pertussis toxin (PT) 100 ng ml^{-1}; then the medium was drawn off and the cells were stimulated with bradykinin. The Ca^{2+}-free medium with or without EGTA (2 mM) added before stimulation with BK was not changed. The BK stimulation was stopped at the indicated times by adding 10% perchloric acid and then cells were kept on ice for 15 min. After neutralization with ice cold 1.5 M KOH, the samples were centrifuged at 2000 g for 15 min at 4°C. The supernatants were kept cold in ice and aliquots of

100 µl were used to measure IP3 with the [^{3}H]-D-myo-inositol 1,4,5-trisphosphate (IP3) RIA kit system (Amersham). The sensitivity range was from 0.5 to 75 pmol ml^{-1}, with 8 and 12% intra and inter-assay variation coefficients respectively.

Statistical analysis was performed with Student's t test and in all comparisons differences were considered significant at $P < 0.05$.

Drugs

Peptides were dissolved in water and 10^{-3} M stock solutions were stored frozen at −70°C. Peptides and drugs for these studies came from the following sources: collagenase (Boehringer Mannheim), penicillin, streptomycin, mytomycin, RPMI, foetal calf serum, D-valine medium, bradykinin (BK), lysyl-bradykinin (LBK), angiotensin II, vasopressin, PGE$_2$, EDTA, EGTA, benzamidine, leupeptin, pertussis toxin, polyethylenimine (Sigma, St. Louis, MO, U.S.A.), myosin antibody (Bio-Yeda), factor VIII antigen (Behring), NBD-phallicidin (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin) was purchased from Molecular Probe; all the bradykinin agonists and antagonists were synthesized by the department of Pharmacology of Sherbrooke University.

Results

Binding studies

As shown in Figure 1, binding with increasing amounts of [^{125}I-Tyr^0]-BK on the mesangial cell membrane gave a maximum number of binding sites (B_{max}) of 73 ± 8 fmol mg^{-1} protein with a dissociation constant (K_d) of 3.7 ± 0.8 nM. The Scatchard plot (insert Figure 1) was linear and the Hill coefficient was not different from unity (0.96 ± 0.04), indicating that the [^{125}I-Tyr^0]-BK bound to only one class of binding site in the range of concentrations tested up to 10 nM. The specificity of [^{125}I-Tyr^0]-BK binding was tested by incubation with various agonist and antagonists (Figure 2). From the inhibition studies we deduced the following order of potency: BK > D-Arg-Hyp^3-D-Phe^7-BK > LBK and the respective IC$_{50}$ values are 0.18 ± 0.03; 0.45 ± 0.04; 37 ± 5 nM. The BK$_1$ agonist, des-Arg^9-BK, was without effect on [^{125}I-Tyr^0]-BK binding. The binding of [^{125}I-Tyr^0]-BK was not inhibited by angiotensin II (10^{-4} M), vasopressin (10^{-4} M) or atrial natriuretic factor (10^{-4} M).

D-myo-Inositol (1,4,5) phosphate production

In Figure 3, the time course of IP3 formation following stimulation with 0.1 µM BK demonstrated a rapid increase from a basal level of 64 ± 14 pmol mg^{-1} protein to a maximal value
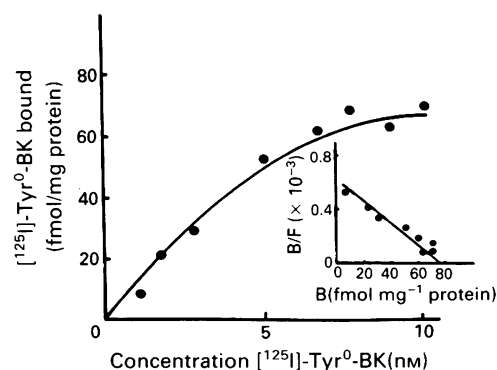
Figure 1 Binding curve and Scatchard analysis (inset) of [^{125}I]-Tyr^0-bradykinin to membrane from mesangial cells. Each point is the mean of triplicate determinations. Data are representative of those obtained in three separate experiments.
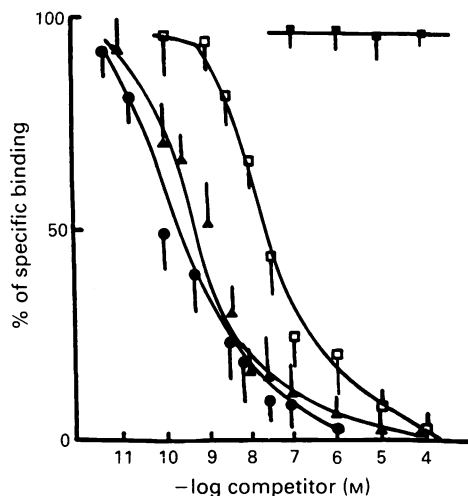
Figure 2 Inhibition of [^{125}I]-Tyr^0-bradykinin binding to membrane from mesangial cells by bradykinin (●), D-Arg-Hyp^3-D-Phe^7-bradykinin (▲), lysyl-bradykinin (□), and Des-Arg^9-bradykinin (■). Each point is the mean of triplicate determinations. Data are representative of those obtained in three separate experiments.

of 175 ± 25 pmol mg^{-1} protein which was reached after only 20 s. Return to basal value was observed within 2 min. Bradykinin induced a dose-dependent stimulation in IP3 formation (Figure 4). The maximal stimulation was achieved with 1 μM BK and the EC$_{50}$ was between 10^{-8} and 10^{-9} M BK.

Pretreatment with pertussis toxin (100 ng ml^{-1}) for 4 h was without effect on BK-induced IP3 formation. In contrast, pretreatment with PMA for 12 h potentiated the BK stimulating effect by about 25% (Figure 3).

When the cells were incubated in a Ca^{2+}-free medium (Figure 5), no change in IP3 formation in response to 0.1 μM BK was observed when compared to the results obtained with a calcium containing medium (Figure 3). However, addition of EGTA to the Ca^{2+}-free medium completely abolished the BK-induced IP3 stimulation (Figure 5).

Incubation of the cells in the presence of the BK$_2$ antagonist, D-Arg-Hyp^3-D-Phe^7-BK (10 μM) inhibited the stimulating effect of 0.1 μM BK. The BK$_2$ antagonist alone did not demon-
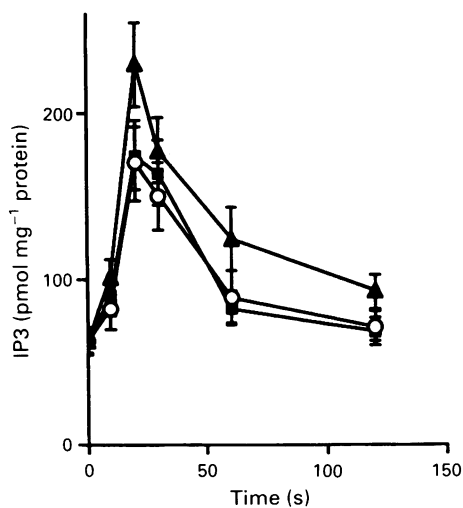
Figure 3 Kinetics of the production of inositol (1,4,5) trisphosphate (■) and effect of pretreatment with pertussis toxin (○) and phorbol 12-myristate 13-acetate (PMA, ▲) on bradykinin (0.1 μM)-stimulated inositol (1,4,5) trisphosphate formation in cultured rat mesangial cells. Bradykinin was added at time zero and the reaction was stopped at the indicated time. Each point is the mean of triplicate determinations. Data are representative of those obtained in three separate experiments.
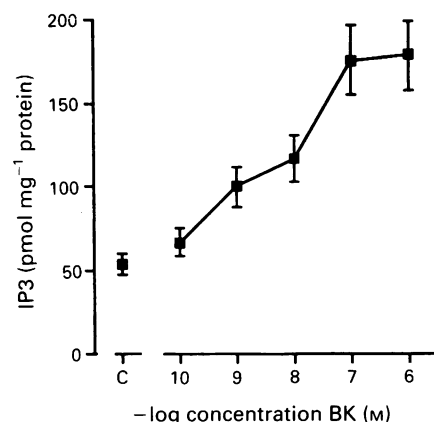

Figure 4 Dose-response curve for bradykinin (BK)-stimulated inositol (1,4,5) trisphosphate formation in cultured rat mesangial cells. C (control value). Cells were exposed to bradykinin for 20 s. Each point is the mean of triplicate determinations. Data are representative of those obtained in three separate experiments.

strate any stimulating or inhibiting effect on IP3 formation (Figure 6). The BK$_1$ antagonist des-Arg^9-Leu^8-BK had no inhibitory effect on the stimulating effect on IP3 formation.

Discussion

The present results show that rat mesangial cells specifically bind bradykinin; this specific binding is coupled to phosphoinositide turnover. Although some of these results could have been expected from previous studies (Baylis *et al.*, 1976; Kremer *et al.*, 1987) the presence of bradykinin binding and the functional role of such binding were not demonstrated until now.

The pharmacological profile of the binding, as well as the inhibition of IP3 formation by D-Arg-Hyp^3-D-Phe^7-BK, strongly suggest the involvement of a BK$_2$-kinin receptor. The kinin receptors have been classified, according to the affinity of agonists and antagonists, into two classes named BK$_1$ and BK$_2$ (Régoli & Barabé, 1980). Specific binding with des-Arg^9-BK reveals the presence of a BK$_1$-kinin receptor, while bradykinin is the most specific agonist of the BK$_2$ receptor. Recently, on the basis of agonists and antagonists activities on uterine and ileal contractions, Braas *et al.* (1988), have suggested the presence of multiple bradykinin-BK$_2$-receptor subtypes. The present data are consistent with our previous
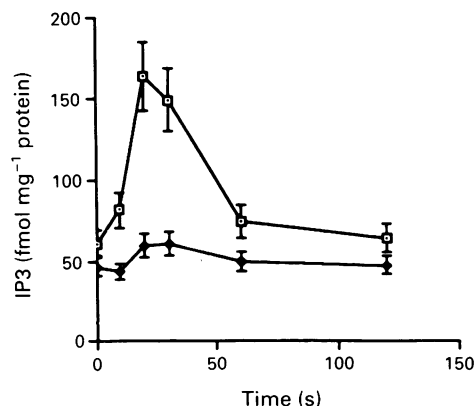
Figure 5 Effect of calcium on inositol (1,4,5) trisphosphate formation in bradykinin-stimulated cultured rat mesangial cells. Cells were preincubated in Ca^{2+}-free medium alone (□), or with EGTA (●) for 5 min before stimulation with 0.1 μM bradykinin which was added at time zero and the reaction was stopped at the indicated time. Each point is the mean of triplicate determinations. Data are representative of those obtained in three separate experiments.
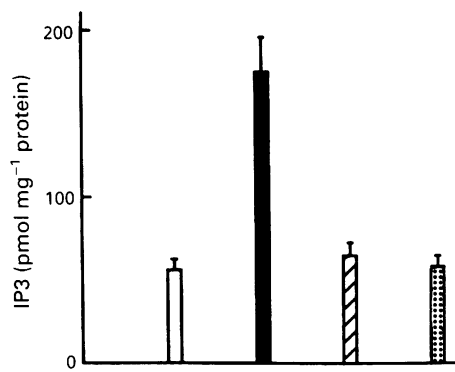
Figure 6 Inhibition of bradykinin-stimulated inositol (1,4,5) tris-phosphate (IP3) formation by incubation with D-Arg-Hyp^3-D-Phe^7-bradykinin. Open column: control; solid column: bradykinin 0.1 μM; hatched column: bradykinin 0.1 μM + D-Arg-Hyp^3-D-Phe^7-bradykinin 10 μM; (stippled column) D-Arg-Hyp^3-D-Phe^7-bradykinin 10 μM. These data are representative of those obtained in three independent experiments.

findings on the glomerular membrane (Bascands et al., 1989), but also provide a possible molecular support to explain the reduction of the ultra-filtration coefficient following intra-renal administration of BK (Baylis et al., 1976). Very recently, it has been demonstrated that bradykinin stimulates phos-phoinositide breakdown in intact rat glomeruli (Sekar et al., 1990). However, the experiments were conducted with crude intact glomeruli which include mesangial, endothelial and epi-thelial cells. In other regions of the kidney or in other organs, specific BK-binding sites have been identified in rat intestinal membranes (Cox et al., 1986), in rat duodenum (Boschcov et al., 1984; Paiva et al., 1989), in epithelial (Portilla & Morriss-on, 1986) and endothelial cells (Lambert et al., 1986). There-fore in intact glomeruli, mesangial cells may not be the sole target for BK. In addition, we found that BK potentiates IP3 formation by 173 ± 15% within 20 s, whereas on intact glo-meruli (Sekar et al., 1990) only a 47 ± 10% increase in total inositides was observed within 5 min in response to the same concentration of BK (0.1 μM). Although different method-ologies are used to assess inositide breakdown, it should be noted that intact glomeruli contain kininase II (Chancel et al., 1987) which could be responsible for a bradykinin degrada-tion that was not assessed in the present investigation (Sekar et al., 1990). All these aspects may explain the difference in the

intensity of the BK-induced IP3 formation between our results and those of a recent study performed on intact glo-meruli (Sekar et al., 1990).

In the present study it was also shown that incubation of the cells in virtually Ca^{2+}-free medium did not inhibit IP3 formation. In most cell systems IP3 formation is considered to be extracellular Ca^{2+}-independent. However, addition of EGTA to the medium caused a 90% reduction in IP3 forma-tion suggesting that slight trans-membrane Ca^{2+} efflux from the cells, sufficient to allow IP3 formation, is counteracted by the presence of EGTA. Such a Ca^{2+}-dependent IP3 formation has previously been reported in neuroblastoma-glioma hybrid cells (Fu et al., 1988).

Furthermore, the results obtained in this study show that long term pretreatment of the mesangial cells with PMA potentiates, by 25%, the BK-stimulated effect on IP3 forma-tion, suggesting that this effect is partly inhibited by protein kinase C (PKC). Long term pretreatment with PMA for 12 h results in a complete desensitization of PKC (Rodriguez-Pena & Rozengurt, 1984) and in this case the response to BK is increased. In contrast, short term exposure to PMA activates PKC transiently and may decrease the BK-stimulating effect as described recently in intact glomeruli (Sekar et al., 1990). Such a negative feed-back of PKC has also been demon-strated for IP3 production in response to angiotensin II (Pfeilschifter, 1986) and vasopressin (Troyer et al., 1988). On the other hand, we found that the release of IP3 is mediated by a pertussis-toxin-insensitive pathway confirming a previous report (Kremer et al., 1987). Although the present report con-tains the first demonstration of BK-induced IP3 formation in mesangial cells, a similar effect has already been reported in other regions of the kidney: in medullary slices (Speziale et al., 1985) in epithelial MDCK cells (Portilla & Morrisson, 1986) and in papillary collecting tubules (Shayman & Morrisson, 1985). In non-renal cell lines, there is also evidence that brady-kinin stimulates phosphoinositides as in Swiss 3T3 fibroblasts (Burch & Axelrod, 1987), in bovine endothelials cells (Martin et al., 1989) and in neuroblastoma cells (Fu et al., 1988).

Therefore, as in other cells, BK induces IP3 formation which appears to be under negative control by PKC. In con-clusion, IP3 formation in mesangial cells is induced by a wide variety of effectors which are all vasoactive peptides such as angiotensin II, vasopressin and now bradykinin. Furthermore the presence of such a large number of receptors linked to IP3 formation and smooth muscle-like activity, suggests the coexistence of different pathways for cell contractility.

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