Vascular mode of action of kinin B₁ receptors and development of a cellular model for the investigation of these receptors

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1 Kinins exert a contractile effect on rabbit aortic rings via the stimulation of B₁ receptors. Des-Arg⁹-bradykinin (BK) is more potent than BK on this receptor type. The mode of action of des-Arg⁹-BK on rabbit aortic tissue has been studied by both the aortic ring contractility assay and a cellular model using cultured aortic smooth muscle cells (SMCs).

2 The des-Arg⁹-BK-induced contractions in rabbit aortic rings were unaffected by pretreatments with nifedipine, indomethacin, REV-5901 (a 5-lipoxygenase blocker) and LY-83583 (a guanylyl cyclase inhibitor); however, the protein kinase inhibitors H-7 and H-9 significantly reduced the maximal effect of des-Arg⁹-BK.

3 The contractile responses to des-Arg⁹-BK in calcium-free Krebs solution were slightly but not significantly attenuated in amplitude, as compared to paired control tissues bathed in Krebs solution, and sustained plateaus of contraction were observed in the absence of Ca²⁺. However, Ca²⁺ replenishment further increased the kinin-induced contraction measured in Ca²⁺-free bathing fluid.

4 Despite the lack of evidence of a mediating role for prostaglandin in the mechanical response to des-Arg⁹-BK, the kinin stimulated the release of prostacyclin from rabbit aorta rings measured as immunoreactive 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}).

5 Smooth muscle cells (SMCs) derived from the rabbit aorta exhibit functional responses to des-Arg⁹-BK in acute release of 6-keto-PGF_{1α} and of inositol phosphate turnover which were inhibited by pretreatment with the B₁ receptor antagonist, Lys[Leu⁸]des-Arg⁹-BK, but not by the B₂ receptor antagonist, Hoe-140. Preincubation of the cells with interleukin-1 (IL-1) 20 h before stimulation with the kinin had no effect on basal inositol phosphate turnover, but potentiated the acute effect of des-Arg⁹-BK.

6 These results suggest that second messengers derived from the action of phospholipase C are produced by SMCs when B₁ receptors are activated in rabbit aortic tissue. Intracellular calcium stores are primarily mobilized by des-Arg⁹-BK, although receptor-controlled calcium influx has not been ruled out, and may contribute to initiate the contractile responses. The maintenance of the contractile state involves protein kinase C activity and is consistent with a current model of SMC function. The cell model retains some of the cardinal properties of B₁ receptor-mediated vascular responses: endothelium-independent PGI₂ release and up-regulation by the cytokine IL-1. PGI₂ is not involved in the mechanical response, possible because the rabbit aorta is refractory to this prostaglandin.

Keywords: des-Arg⁹-bradykinin; B₁ receptors for kinins; rabbit aortic preparation; signal transduction

Introduction

While the classification of receptor for bradykinin (BK) still awaits the molecular cloning of all the relevant receptor molecules, considerable pharmacological evidence supports the broad classification into the B₁ and B₂ types (Regoli & Barabé, 1980; Farmer & Burch, 1992). The B₁ receptor is stimulated selectively by kinin fragments of BK and Lys-BK without the C-terminal arginine residue such as des-Arg⁹-BK and Lys-des-Arg⁹-BK. The development of competitive, potent and selective antagonists (prototype [Leu⁸]des-Arg⁹-BK) has consolidated the pharmacological identity of the receptors of the B₁ type (Regoli *et al.*, 1977). Agonists and antagonists for B₁ receptors are conveniently assayed on the rabbit aortic preparation, which exhibits a prostaglandin-independent contractile response to kinins solely via this receptor type (Regoli *et al.*, 1977). The B₂ receptor type is optimally stimulated by the native kinins, BK or Lys-BK, and is antagonized by numerous sequence-related synthetic peptides, of which Hoe-140 is an excellent example (Hock *et al.*, 1991). Hoe-140 is a very high affinity antagonist that has no affinity for the B₁ receptors, and hence, does not inhibit kinin-induced contractions in the rabbit isolated aorta (Hock

et al., 1991). Conversely Hoe-140 is probably effective on all preparations containing the B₂ receptors, providing some uniformity to this pharmacological entity. The two kinin receptors cloned to-date from rat and human tissues belong to the B₂ class; they are highly homologous and the human type can be functionally inhibited by Hoe-140, but not by [Leu⁸]des-Arg⁹-BK (McEachern *et al.*, 1991; Hess *et al.*, 1992).

The B₁ receptors for kinins are usually not sufficiently dominant to account for the cardiovascular effects of kinins, such as hypotension, vasodilatation and increased vascular permeability. However, careful analysis has revealed that they account for a significant part of the effect of kinins in normal dogs (Toda *et al.*, 1987; Müller-Schweinitzer, 1988; Rhaleb *et al.*, 1989; Lorti *et al.*, 1992), and that they co-exist with B₂ receptors following an apparent up-regulation process in the cardiovascular system of rabbits or pigs injected with bacterial material or experiencing experimental arthritis (Regoli *et al.*, 1981; Drapeau *et al.*, 1991; Siebeck *et al.*, 1989; Farmer *et al.*, 1991). In chronic models of inflammation in the rat, kinin-mediated hyperalgesia is reportedly largely dependent on B₁ receptors (Perkins *et al.*, 1992). We have shown that the contractile response to B₁ receptor agonists is acquired *in vitro* in the prototype preparation for B₁ receptor definition, the rabbit isolated aorta; this process is dependent on protein synthesis and can be further amplified by the

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cytokine interleukin-1 (IL-1) (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988; 1991). In other preparations, such as the rabbit mesenteric and dog renal arteries, the dominant relaxation response to the stimulation of B₁ receptors is a prostaglandin-dependent and endothelium-independent process (Churchill & Ward, 1986; deBlois & Marceau, 1987; Rhaleb *et al.*, 1989). A significant portion of the hypotensive effect of B₁ agonists in lipopolysaccharide-treated rabbits is also accounted for by prostaglandin release (Drapeau *et al.*, 1991).

Data on the cardiovascular effects dependent on the B₁ receptors are lacking because no systematic studies on the signal transduction pathways or on receptor binding assay have been reported to date. The direct contractile effect of kinins on the rabbit isolated aorta is unaffected by indomethacin, endothelium removal and tyrosine kinase inhibitors (deBlois *et al.*, 1992), but is partially inhibited by the removal of extracellular calcium, or by treatments with NiCl₂ (used as a Ca²⁺ channel inhibitor), or by protein kinase C inhibitors (Calixto & Medeiros, 1992). In the porcine isolated coronary artery, the contractile effect of des-Arg⁹-BK did not occur simultaneously with a significant change in membrane potential (Beny *et al.*, 1987). These observations are compatible with the activation of the phospholipase C signalling pathway and also, possibly, with the activation of receptor-controlled calcium channels following B₁ receptor stimulation. Direct evidence of phospholipase C activation by B₁ receptor stimulation was obtained in cultured rat mesangial cells, one of the numerous cultured cell types that express this pharmacological entity (Issandou & Darbon, 1991). Agonists that activate membrane associated tyrosine kinase activities, such as insulin and epidermal growth factor, have been reported to act synergistically with B₁ receptor agonists in the rat mesangial cells and in rabbit aortic strips, respectively (Issandou & Darbon, 1991; deBlois *et al.*, 1992).

The present study has been undertaken to characterize further the mode of action of B₁ receptor agonists in the rabbit aortic preparation. Ultimately, we hope to develop a cellular model that would retain the essential characteristics of the vascular preparations to study the cardiovascular actions of B₁ receptor-mediated effects of kinins. Such a model would allow the analysis of possible signalling pathways (calcium, eicosanoids and phospholipase C) and of receptor population regulation subsequent to immunological stimulation.

Methods

Organ bath pharmacology of B₁ receptors

The thoracic aorta was isolated from New Zealand White rabbits of either sex (1.5–2 kg). The vessels were cut into rings (2–3 mm width), suspended between a metal hook and a thread loop under a basal tension of 2 g in 5 ml organ chambers containing oxygenated (95% O₂:5% CO₂) and warmed (37°C) Krebs solution (Marceau *et al.*, 1991). Their responses to agents were recorded isometrically as described previously (Bouthillier *et al.*, 1987).

In order to analyze the mechanism of the contractile effect of the selective B₁ receptor agonist des-Arg⁹-BK, a nearly maximal concentration (1.7 µM) of this agent was applied to tissues after 3.5 to 4.5 h of equilibration. This time was kept constant in treated tissues and their paired controls because the maximal effect of this kinin changes considerably as a function of the incubation time. The mechanism for the increase of tissue response to B₁ receptor agonists has been documented (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988; 1989; 1991). Inhibitory drugs were applied 30 min before the kinin and then continuously in treated groups; paired control tissues were used to evaluate the effect of the drug vehicle, if any. The same tissues were also challenged at later times with other submaximal contractile stimuli for comparison purpose: KCl (30 mM) and phorbol myristate acetate (PMA;

1.1 µM). The inhibitory drugs under study were nifedipine, an L-type Ca²⁺ channel blocker (Murad, 1990), the protein kinase inhibitors H-7 and H-9 (Hidata *et al.*, 1984), indomethacin, the 5-lipoxygenase blocker (REV-5901 (McMillan & Walker, 1992) and the guanylyl cyclase inhibitory LY-83583 (Mulsch *et al.*, 1989). The concentrations of the drugs were chosen on the basis of their reported activity in various *in vitro* systems and, when possible, relevant controls were obtained in rabbit aortic tissue. In some experiments involving H-7, full cumulative concentration-effect curves were established for KCl and des-Arg⁹-BK to document further the effect of the kinase inhibitor on a wide range of contraction amplitudes for both agents. Another protocol consisted of replacing the Krebs solution by a calcium-free formulation of Krebs solution, 5–10 min before each contractile stimulation; this was done to evaluate the contribution of extracellular calcium to the effect of each type of stimulation. Between tests, the tissue preparation was maintained in regular Krebs solution with calcium to avoid depletion of the intracellular pools.

In some experiments, the possible stimulation of eicosanoid release by des-Arg⁹-BK was tested by measuring the concentration of 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}), the major cyclo-oxygenase product in this tissue (Förstermann *et al.*, 1984), in the bathing fluid. Some tissues were exposed to indomethacin (2.8 µM) 30 min before the effect of des-Arg⁹-BK was examined. The aortic rings were washed and three aliquots of the bathing fluid were successively taken at 10 min intervals. Immediately after the second sampling, des-Arg⁹-BK (1.7 µM) was applied to each tissue. 6-Keto-PGF_{1α} release results are expressed as the mass secreted per aortic ring during two consecutive 10-min periods, firstly a basal secretion period followed by a treatment period. These values were calculated by subtracting the concentration at the beginning of a period from the concentration at the end of that period. The prostaglandin concentration was determined by a specific radioimmunoassay (Lebel & Grose, 1986).

Cell culture

Several distinct lines of rabbit smooth muscle cells were established from rabbit aortae aseptically removed from freshly killed animals. The tissues (approximate weight 200 mg) were incubated at 37°C for 1.5 h in medium 199 (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco), antibiotics (streptomycin, 50 µg ml⁻¹, and penicillin, 50 u ml⁻¹; Gibco), bacterial collagenase (type IA, 3 mg ml⁻¹) and elastase (type III, 1 mg ml⁻¹); all enzymes were obtained from Sigma. Dispersed cells and partly digested tissue fragments were centrifuged and resuspended in medium 199 supplemented with 10% FBS and antibiotics. This suspension was seeded directly in 25 cm² flasks coated with 0.2% gelatin. Cells were passaged on gelatin-coated surfaces at confluency with a brief trypsin (0.05%)-EDTA (0.53 mM) treatment. The cells were studied at passages 1 to 7 only; their identity as smooth muscle cells was checked by indirect immunofluorescence for α-actin, a smooth muscle marker (monoclonal antibody 1A4 from Sigma) with a Bio-Rad MRC-600 scanning confocal microscope.

Experiments were conducted with the same type of cells to evaluate the turnover of phosphoinositides and the production of prostaglandin in response to B₁ receptor stimulation.

Inositol phosphate production

Labelling and stimulation of rabbit aorta smooth muscle cells were carried out according to Balla *et al.* (1988) with some modifications. Briefly, cells were seeded at a density of 2.5 × 10⁵ in wells of 2 cm² in the culture medium described above and cultured for 24 h. The cells were then washed three times with serum-free medium. The incorporation medium (500 µl) consisted of Medium 199 containing 20 µCi ml⁻¹ of myo-[³H]-inositol (Amersham, specific activity 82 Ci

mmol⁻¹) and IL-1 β (5 ng ml⁻¹), when indicated, was added to the wells for a labelling period of 16–20 h. After incubation at 37°C in a humidified atmosphere containing 5% CO₂, each well was washed twice with Earle's balanced salt solution (EBSS). The cells were further incubated for 20 min in EBSS with 10 mM LiCl and with a kinin antagonist, when indicated. The cells were then challenged with des-Arg⁹-BK (1.7 μ M) or its vehicle for exactly 5 min. Stimulations were terminated by the addition of perchloric acid (final concentration 5%).

Inositol phosphates were extracted and separated as previously described (Guillemette *et al.*, 1989). At the end of the experiment, the cells were kept on ice for 30 min and then scraped from the wells and transferred into tubes. The wells were rinsed with additional perchloric acid (5% w/v) and the resulting media was centrifuged at 10,000 g for 5 min. Perchloric acid was extracted from the supernatant with a 1:1 mixture of Freon and tri-*n*-octylamine (Downes *et al.*, 1986; reagents from Sigma). After neutralization, the samples were applied to anion-exchange resin (Dowex AG 1-X8 formate, 200–400 mesh, Bio-Rad No. 140–1450) columns (1 ml bed volume) for the separation of the inositol phosphates according to Berridge *et al.* (1983). The inositol phosphates were sequentially eluted with 3 $\times$ 5 ml of distilled water (free inositol), 3 $\times$ 5 ml of 0.2 M ammonium formate/0.1 M formic acid (inositol monophosphates, IP₁), 3 $\times$ 5 ml of 0.5 M ammonium formate/0.1 M formic acid (inositol biphosphates, IP₂), 3 $\times$ 5 ml of 0.8 M ammonium formate/0.1 M formic acid (inositol trisphosphates, IP₃) and 3 $\times$ 5 ml of 1.0 M ammonium formate/0.1 M formic acid (inositol tetrakisphosphates, IP₄). The fractions were collected, mixed with Ecolite + scintillation fluid (ICN Biomedicals, Irvine CA, U.S.A.) and counted for radioactivity in a liquid scintillation counter (Beckman LS 8000).

Smooth muscle cells were used to evaluate the kinin-induced release of 6-keto-PGF_{1 α} . Cells were seeded in gelatin-coated 12-well plates (Linbro, Rockville MD, U.S.A.; well diameter 24 mm) at a density of 3 $\times$ 10⁴ per well and cultured for 24 h in the complete medium described above. The culture medium was then removed, the wells were washed once and replaced with 1 ml of equilibrated (37°C, 5% CO₂) EBSS containing the test substances (a kinin agonist, antagonist or both together). After a 20 min incubation period, the supernatants were collected for measurement of 6-keto-PGF_{1 α} as described above.

Drugs

The following drugs were purchased from Sigma (St-Louis MO, U.S.A.): nifedipine, H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine), H-9 (N-(2-aminoethyl)-5-isoquinolinesulphonamide), indomethacin and PMA (phorbol 12-myristate 13-acetate). REV-5901 (α -pentyl-3-(2-quinolinylmethoxy)-benzene methanol, *para* isomer) was purchased from Biomol (Plymouth Meeting PA, U.S.A.) and LY-83583 (6-anilino-5,8-quinolinedione), from Calbiochem (La Jolla CA, U.S.A.). Hoe-140 (D-Arg[Hyp³,Thi⁵,D-Tic⁷,Oic⁸]-BK; icatiban) was a gift from Dr K. Wirth (Hoechst AG, Frankfurt, Germany). Des-Arg⁹-BK was purchased from Bachem (Torrance CA, U.S.A.) and Lys[Leu⁸]des-Arg⁹-BK was synthesized in our laboratories (Drapeau & Regoli, 1988). Human recombinant interleukin-1 β was a gift of Dr D.E. Tracey (Upjohn Co., Kalamazoo MI, U.S.A.).

Results

Contractility assays

Comparative experiments were performed with des-Arg⁹-BK, a very selective B₁ receptor agonist, and with other contractile agents in rabbit aortic rings (Table 1). A nearly maximal concentration (ED₉₅) of the kinin was chosen for these experiments, whereas the effect of KCl (30 mM) and of PMA (1.1 μ M) was submaximal in each case. Two or three contractile responses were recorded at the same time for control and treated rings after at least 3.5 h of equilibration; this pairing is necessary because the effect of des-Arg⁹-BK varies as a function of the time of *in vitro* incubation and because there is a large inter-animal variation for these responses (Bouthillier *et al.*, 1987). Inhibitory drugs were applied 30 min before recording contractile responses to the kinin.

The voltage-operated calcium channel blocker, nifedipine, had no significant effect on the contractile response to des-Arg⁹-BK. In the same tissues, it inhibited very extensively the contractile effect of the depolarizing agent KCl, providing an internal control of the effectiveness of the drug at the concentration tested. Protein kinase inhibitors were tested in particular to evaluate the possible role of protein kinase C following the stimulation of B₁ receptors. Both protein kinase blockers H-7 (10 μ M) and H-9 (30 μ M) partially inhibited the

Table 1 Pharmacological analysis of the mode of action of the B₁ receptor agonist des-Arg⁹-BK in the rabbit aortic preparation

Treatment	n	Contractile responses (g)		
		des-Arg ⁹ -BK (1.7 μ M)	KCl (30 mM)	PMA (1.1 μ M)
Nifedipine 100 nM	6	0.84 $\pm$ 0.09	0.16 $\pm$ 0.02****	NT ^b
Vehicle (ethanol)	6	1.03 $\pm$ 0.08	2.71 $\pm$ 0.26	NT
H-7 10 μ M	8	1.0 $\pm$ 0.3*	3.3 $\pm$ 0.3	0.14 $\pm$ 0.01***
Vehicle (saline)	8	1.7 $\pm$ 0.3	3.6 $\pm$ 0.1	0.27 $\pm$ 0.02
H-9 30 μ M	6	0.57 $\pm$ 0.15**	2.65 $\pm$ 0.34	0.09 $\pm$ 0.01***
Vehicle (HCl)	6	1.34 $\pm$ 0.17	4.10 $\pm$ 0.67	0.30 $\pm$ 0.03
Indomethacin 2.8 μ M	14	0.70 $\pm$ 0.12	2.87 $\pm$ 0.35	NT
Vehicle (Na ₂ CO ₃)	14	0.89 $\pm$ 0.19	2.78 $\pm$ 0.33	NT
REV-5901 500 nM	6	1.19 $\pm$ 0.29	3.78 $\pm$ 0.49	NT
Vehicle (ethanol)	6	0.98 $\pm$ 0.09	3.24 $\pm$ 0.47	NT
LY-83583 10 μ M	5	0.74 $\pm$ 0.22	3.03 $\pm$ 0.49	NT
Vehicle (DMSO)	5	0.79 $\pm$ 0.02	3.13 $\pm$ 0.09	NT
Calcium-free Krebs ^c	10	1.39 $\pm$ 0.48	0.24 $\pm$ 0.04***	NT
Control	10	1.63 $\pm$ 0.30	4.62 $\pm$ 0.30	NT

*Values in treated groups were compared to controls from the same animals by Student's *t* test for paired samples: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

^bNT, not tested.

^cTissues were exposed to calcium-free Krebs solution only 10 min before each recording and were then placed in normal Krebs for recovery.

Figure 1 Effect of H-7 (10 μ M) on the cumulative concentration-effect curves for KCl (a, c) and des-Arg⁹-BK (b, d) in rabbit aortic rings. In (a) and (b) results are expressed in g of developed contraction; the same data are plotted as a percentage of the maximal response in (c) and (d). Abscissa scale: $-\log$ of molar concentration. Values are the means $\pm$ s.e.mean of 6 determinations for KCl, 8 for des-Arg⁹-BK. Open symbols: values recorded in the presence of H-7; closed symbols, controls. Student's *t* test for paired samples was used to assess the effect of H-7: **P* < 0.05; ***P* < 0.01.

effect of des-Arg⁹-BK and of PMA, but not that of KCl. These results may indicate a role for one of the susceptible protein kinases in the contractile response to des-Arg⁹-BK. The selectivity of the effect of the drug H-7 (10 μ M) was further analyzed by constructing full cumulative concentration-effect curves for des-Arg⁹-BK and KCl in its presence (Figure 1). The contractile effect of all concentration levels of the kinin were significantly depressed by H-7 (Figure 1b), whereas only one of the smaller concentration of KCl was affected by the drug (Figure 1a). The EC₅₀ for KCl was not significantly affected by H-7 ($2.7 \pm 0.1 \times 10^{-2}$ M in the presence of H-7; control value $2.5 \pm 0.05 \times 10^{-2}$ M). H-7 shifted the curve slightly to the right for the kinin (EC₅₀ of $1.9 \pm 0.3 \times 10^{-7}$ M in the presence of H-7; control value of $1.1 \pm 0.2 \times 10^{-7}$ M; *P* < 0.01 by Student's *t* test for paired samples).

As reported previously (Regoli *et al.*, 1977; deBlois *et al.*, 1992), indomethacin did not inhibit the effect of des-Arg⁹-BK in the rabbit aortic preparation; the drug was also not

effective on the KCl-induced contractions (Table 1). The 5-lipoxygenase blocker, REV-5901, failed to influence the effects of the contractile agents, des-Arg⁹-BK or KCl. The inhibitor of the soluble form of guanylyl cyclase, LY-83583, also failed to inhibit the contractile effect of any of the two stimuli at a concentration that reportedly inhibits guanosine 3':5'-cyclic monophosphate (cyclic GMP) accumulation in the acetylcholine-stimulated rabbit aorta (Diamond, 1987).

The major eicosanoid produced by the isolated aorta, either in the basal or stimulated conditions is PGI₂ (Förstermann *et al.*, 1984). We have measured the concentration of immunoreactive 6-keto-PGF_{1 α} , the PGI₂ breakdown product, in the bathing fluid of rabbit aortic rings challenged with des-Arg⁹-BK (Table 2). The paired tissues exposed to indomethacin are the same preparations for which the mechanical responses are reported in Table 1. Des-Arg⁹-BK treatment increased sharply the release of the eicosanoid during the experimental 10 min period, as compared to a similar basal release period. However, tissues treated with indomethacin released less 6-keto-PGF_{1 α} in the basal period and did not respond to the kinin stimulation. This suggests that eicosanoid release occurs in the rabbit isolated aorta in response to B₁ receptor stimulation, but does not affect the mechanical response to the same stimulus.

A group of experiments was designed to evaluate the role of extracellular calcium in the B₁ receptor-mediated contractile events. Tissues incubated in Krebs solution were rinsed extensively with calcium-free Krebs 10 min before their response to des-Arg⁹-BK or to control stimulus was recorded (Table 1, statistical analysis). After the maximal effect was recorded, the tissues were washed and further incubated in regular Krebs solution with calcium. This procedure was developed to avoid long-term leakage of intracellular calcium

Figure 2 Representative tracings of the contraction profiles recorded from different aortic rings from a single rabbit. Tissues were successively stimulated with des-Arg⁹-BK (1.7 μ M) and KCl (30 nM). (a) Control tracing, in regular Krebs solution; (b) effect of calcium removal 5 min before pharmacological stimulation; (c) effect of calcium concentration restoration (2.5 mM) on contraction developed in calcium-free Krebs. Closed symbols refer to stimulus application and open symbols, to the first of a series of tissue washing with normal Krebs solution. Abscissa scale: time; ordinate scale: isometric contraction, g.

Table 2 Release of 6-keto-prostaglandin F_{1 α} (6-keto-PGF_{1 α}) from rabbit aortic rings during two consecutive 10 min basal and des-Arg⁹-BK-stimulated (1.7 μ M) periods

Pretreatment	n	6-Keto-PGF _{1α} (pg released/10 min)	
		Basal period	des-Arg ⁹ -BK
None (control)	14	39 $\pm$ 12	211 $\pm$ 68**
Indomethacin 2.8 μ M	14	16 $\pm$ 4	8 $\pm$ 2

*Values obtained in the treated periods were compared to those measured in the control periods by Student's *t* test for paired samples: **P* < 0.05. The mechanical responses of these tissues to des-Arg⁹-BK are documented in Table 1.

stores and is validated by the extensive inhibition of KCl-induced contraction, a stimulus which is essentially dependent on extracellular calcium (Cauvin *et al.*, 1983). Under these conditions, there was some inhibition of the maximal effect of des-Arg⁹-BK, although it was not statistically significant in this series of experiments. This protocol is illustrated by representative tracings (Figure 2). It can be seen that tissues contracted with the kinin in the absence of extracellular calcium maintain a good contraction plateau over a 10 min period (Figure 2b). In tissues contracted with des-Arg⁹-BK in the absence of calcium, replenishment of calcium when the effect had developed further increased the contractile response, whereas the KCl-induced contraction was initiated only when Ca²⁺ concentration was restored (Figure 2c). The same experiment conducted in tissues pretreated with nifedipine produced identical results for des-Arg⁹-BK: there was an additional tension that developed following the introduction of Ca²⁺ (tracings not shown).

Cellular model of the vascular effect of des-Arg⁹-BK

Cells derived from the digestion of rabbit aortic tissue without endothelium exhibited a narrow, pointed morphology; when the cultures approached confluence, parallel bundles of cells were observed under phase contrast microscopy (Figure 3a). Most of the cells stained positive for the smooth muscle marker α -actin as cytosolic fibrillar patterns, (Figure 3b,c), but the intensity of the staining was variable from one cell to another.

The cultured smooth muscle cells derived from the rabbit aorta retained the capacity to respond to kinins by increased release of 6-keto-PGF_{1 α} (Figure 4, Table 3). The selective B₁ agonist des-Arg⁹-BK elicited a concentration-dependent stimulant effect, with statistical significance at and above 10 nM (Figure 4). BK was not statistically active in this respect up to 10 μ M. The kinin antagonists Hoe-140 and Lys[Leu⁸]des-Arg⁹-BK had no direct effect on basal 6-keto-PGF_{1 α} release at 1.7 μ M (Table 3). Only the latter antagonist, selective for the B₁ receptors, reduced the influence of des-Arg⁹-BK when co-administered.

Evidence was also obtained for des-Arg⁹-BK-induced IP turnover in this type of cell (Table 4). In experiment 1, des-Arg⁹-BK (1.7 μ M) acutely induced the accumulation of inositol phosphates in cells maintained in both the regular medium, which significantly increased the concentration of IP₂ and IP₄, and in the presence of IL-1 β , which significantly increased levels of IP₁, IP₂ and IP₃. In the absence of the kinin, IL-1 β alone had no effect, but there appears to be an increased incorporation efficiency of *myo*-inositol into the cells. However, IL-1 β pretreatment potentiated significantly the des-Arg⁹-BK effect on the cellular IP₃ concentration. It was previously shown that the cytokine increased the maximal contractile effect of des-Arg⁹-BK considerably in fresh aortic tissue over several hours (deBlois *et al.*, 1988; 1991).

All the cells were pretreated with IL-1 β in experiment 2 to maximize the effect of the kinin. As shown in Table 4, the kinin antagonist Lys[Leu⁸]des-Arg⁹-BK (1.7 μ M) failed to increase IP metabolism significantly in cultured SMCs, but it inhibited the stimulatory effect of des-Arg⁹-BK on IP₁ and IP₃ concentrations, suggesting that the influence of this kinin on phospholipase C activity is related to B₁ receptor-mediated events. The B₂ antagonist, Hoe-140, did not inhibit des-Arg⁹-BK-induced alterations in IP concentrations (Student's *t* test); at 1.7 μ M, the antagonist peptide may have stimulated IP production directly and thus potentiated the kinin-induced IP₂ production, but the changes were not statistically significant.

Discussion

Complementary approaches were used to analyze the mode of action of des-Arg⁹-BK employing the rabbit aortic prep-

Figure 3 Cultured smooth muscle cells from rabbit aortic tissue. (a) Phase contrast appearance passage 1 ($\times 100$); (b and c) indirect immunofluorescence for the smooth muscle specific α -actin (passages 1 and 4, respectively). Horizontal bar: 10 μ m scale.

Figure 4 Concentration-effect relationship for des-Arg³-BK- (●) and bradykinin (BK)-induced (□) production of 6-keto-PGF_{1α} from cultured smooth muscle cells derived from rabbit aortae. Values are the means ± s.e. mean of 15 values composed of data from 6 cell lines (passages 3 to 7). The basal release value (○) is the mean ± s.e. mean of 36 values from the same cell lines. One-way analysis of variance indicated that the groups were not homogeneous ($P < 0.01$). Dunnett's test was then applied to compare the effect of each treatment with the controls (Tallarida & Murray, 1987): * $P < 0.05$; ** $P < 0.01$.

aration, the prototype bioassay for this receptor type (Regoli & Barabé, 1980). Using contractility as the end point, it was shown that rabbit aortic rings are less dependent on extracellular calcium when stimulated with des-Arg³-BK than with KCl. A relatively sustained contraction was observed with the kinin, even in the absence of extracellular calcium in the bathing fluid. Another group of investigators has suggested that the BK-induced contraction, also mediated by B₁ receptors, is highly dependent on extracellular calcium; this conclusion is based on experiments conducted in a Ca²⁺-free physiological medium and on the modest inhibitory effect of NiCl₂ (Calixto & Medeiros, 1992). In that report, however, there was a 30 min pre-incubation period in a Ca²⁺-free buffer before pharmacological stimulation in the form of a cumulative concentration scale was carried out. Our dis-

crepant findings may be due to the selection of a single peptide concentration and of a shorter preincubation period of only 5–10 min; this approach may prevent the time-dependent 'leakage' of intracellular calcium pools, and at the same time was sufficiently effective to inhibit 95% of the contractile effect of KCl, which is a depolarizing stimulus dependent on extracellular Ca²⁺ (Cauvin *et al.*, 1983). Restoration of the Ca²⁺ concentration in a tissue precontracted with des-Arg³-BK in Ca²⁺-free Krebs solution further increased contraction; this experiment suggests that during the des-Arg³-BK-induced stimulations influx of Ca²⁺ occurs. However, this component which was not inhibited by nifedipine, is relatively minor. It may be due to receptor-controlled Ca²⁺ channels not sensitive to dihydropyridines. Nifedipine had no effect on kinin-induced contractions, but it abolished the KCl-induced contraction, which is consistent with the inhibitory effect of this drug on the voltage-operated channels (Murad, 1990). The lack of effect of des-Arg³-BK on membrane potential in porcine coronary arteries has been reported previously (Beny *et al.*, 1987) and is consistent with the lack of effect of nifedipine noted in the present study, and of nicardipine in a recent publication (Calixto & Medeiros, 1992).

Contractility data obtained with aortic rings also suggest the involvement of protein kinase(s) in des-Arg³-BK-induced contraction. Protein kinase C is susceptible to inhibition by both H-7 and H-9 (Hidata *et al.*, 1984) and, accordingly, both compounds suppressed effectively the small contractile effect due to the protein kinase C activator, PMA. Also, the two inhibitors partially but significantly reduced the contractile effect of des-Arg³-BK, suggesting that the signal transduction of B₁ receptors may be physiologically regulated by diacylglycerol derived from the phospholipase C pathway. H-7 exhibited some selectivity in its action as it inhibited only minimally equicontractile concentrations of KCl (Figure 1). Des-Arg³-BK is not likely to increase the intracellular Ca²⁺ concentration in a sustained manner, especially in the absence of extracellular Ca²⁺; however, the observed contraction plateaus are very stable (Figure 2). According to a concept of smooth muscle contraction (Rasmussen *et al.*, 1987), relative to the mechanical response, the rise in intracellular calcium concentration and the resulting activation of the myosin light chain kinase are only transient in SMCs, but these components are necessary to initiate a strong contractile response. Protein kinase C activation is involved in the

Table 3 Pharmacological characterization of the kinin-induced release of 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}) from cultured smooth muscle cells from the rabbit aorta

Agonist	Antagonist	6-keto-PGF _{1α} release (pg/well)	Dunnett's test ^a
None	None	421 ± 27	—
	Lys[Leu ⁸]des-Arg ³ -BK 1.7 μM	472 ± 52	NS
	Hoe-140 1.7 μM	456 ± 66	NS
des-Arg ³ -BK 1.7 μM	None	948 ± 85	< 0.01
	Lys[Leu ⁸]des-Arg ³ -BK 1.7 μM	567 ± 67*	NS
	Hoe-140 1.7 μM	878 ± 78	< 0.01
BK 1.7 μM	None	542 ± 59	NS
	Lys[Leu ⁸]des-Arg ³ -BK 1.7 μM	451 ± 39	NS
	Hoe-140 1.7 μM	629 ± 105	NS

^aValues are the means ± s.e. mean of 9 values composed of triplicate determinations from 3 cell lines (passages 4 to 6), except for the control group (first line), which composed of 26 values from the same cell lines. One-way analysis of variance was performed ($P < 0.01$). Dunnett's test was then applied to compare the effect of each treatment with the baseline prostaglandin release (first line) (Tallarida & Murray, 1987). The effect of each antagonist was compared to the corresponding controls exposed to the same agonist by Student's *t* test (* $P < 0.01$).

Table 4 Inositol phosphates measurements in rabbit aortic smooth muscle cells 5 min after exposure to des-Arg⁹-BK (1.7 μ M), to a kinin antagonist (1.7 μ M of either one), or to the combination of des-Arg⁹-BK and an antagonist^a

Pretreatments ^b	Stimulus	IP ₁	Inositol phosphates (c.p.m. per well) ^c			IP ₄	Cell-associated myo-inositol
			IP ₂	IP ₃			
<i>Experiment 1</i>							
None	Control	2295 ± 459	300 ± 58	188 ± 22	148 ± 9	126869 ± 7021	
None	des-Arg ⁹ -BK	3631 ± 673	785 ± 199 nd	270 ± 40	179 ± 11*	115241 ± 13124	
IL-1β (− 20 h)	Control	1619 ± 356	328 ± 35	155 ± 10	150 ± 14	187081 ± 30785	
IL-1β (− 20 h)	des-Arg ⁹ -BK	5686 ± 882***	1073 ± 182**	518 ± 82***†	231 ± 43	198577 ± 29783†	
<i>Experiment 2</i>							
IL-1β (− 20 h)	Control	1250 ± 173	145 ± 31	88 ± 15	61 ± 11	270932 ± 23808	
IL-1β (− 20 h)	des-Arg ⁹ -BK	2567 ± 437*	298 ± 48	166 ± 29*	84 ± 11	291997 ± 61488	
IL-1β (− 20 h)	Lys-[Leu ⁸]-des-Arg ⁹ -BK	1264 ± 244	203 ± 31	102 ± 10	69 ± 11	284019 ± 44703	
IL-1β (− 20 h)	Hoe-140	1656 ± 318	212 ± 31	118 ± 11	76 ± 9	243001 ± 26440	
IL-1β (− 20 h)	des-Arg ⁹ -BK + Lys-[Leu ⁸]-des-Arg ⁹ -BK	1115 ± 112	174 ± 50	93 ± 16	64 ± 9	267460 ± 23933	
IL-1β (− 20 h)	des-Arg ⁹ -BK + Hoe-140	2354 ± 460	481 ± 80**	175 ± 31*	89 ± 10	304648 ± 64560	
<i>Analysis of variance^e</i>		<i>P</i> < 0.01	<i>P</i> < 0.01	<i>P</i> < 0.01	NS	NS	

^aSmooth muscle cells from different cell lines were used at passage 2, 3 and 4 for experiment 1 (values are the means $\pm$ s.e.mean of 9 values composed of triplicate determinations from 3 cell lines) and 4 or 5 for experiment 2 (values are the means $\pm$ s.e.mean of 12 values composed of triplicate determinations from 4 cell lines).

^bHuman recombinant IL-1 β (5 ng ml⁻¹) was present during the 20 h incubation period with myo-[³H] inositol.

^cIn experiment 1, one of the variables (IL-1 presence) influenced significantly myo-inositol incorporation; therefore results are expressed directly as c.p.m. per well. In experiment 2, IL-1 was uniformly applied and the myo-inositol incorporation was homogeneous in all groups (ANOVA). Therefore the results have been normalized as c.p.m. of IP per 100,000 c.p.m. of the remaining cell-associated myo-inositol in each cell well.

^dFor experiment 1, the effect of des-Arg⁹-BK was compared with controls by Student's *t* test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. The effect of IL-1 pretreatment on experimental values was also assessed by comparing IL-1-treated groups with the corresponding group (with or without kinin challenge) by Student's *t* test: †*P* < 0.05.

^eFor experiment 2, one-way analysis of variance was performed on columns of data (NS, not significant). In three columns the test indicated that groups were significantly different from each other. Dunnett's test was then applied to compare the effect of each treatment with the control cells pretreated with IL-1 β only (first line) (Tallarida & Murray, 1987): **P* < 0.05; ***P* < 0.01.

maintenance of the contractile state, but if it is not preceded by a Ca²⁺ concentration increase, as with PMA, it leads only to a very small and slow developing contraction (Rasmussen *et al.*, 1987).

The use of protein kinase C inhibitors has some limitations, which include lack of selectivity and they may also effect several distinct protein kinases. For example, the affinity of H-9 for rabbit protein kinase C is supported by the use of this drug in affinity chromatography to isolate this kinase from rabbit tissues (Inagaki *et al.*, 1985). However, H-9 and H-7 also inhibit cyclic nucleotide-dependent protein kinases (Hidata *et al.*, 1984). The lack of effect of the guanylyl-cyclase inhibitor LY-83583, as well as the known relaxant effect of both cyclic AMP and cyclic GMP in vascular smooth muscle suggest that the B₁ receptors are not linked directly to cyclic nucleotide metabolism in the rabbit aortic tissue. Several tyrosine kinase inhibitors had no effect on des-Arg⁹-BK-induced contraction in the aortic preparation, but they do inhibit the potentiation of the kinin effect by epidermal growth factor (deBlois *et al.*, 1992). Indomethacin at low concentrations reportedly inhibits a cyclic AMP-dependent protein kinase, in addition to suppressing the synthesis of prostanoids (Kantor & Hampton, 1978). This drug and the 5-lipoxygenase blocker, REV-5901, an inhibitor of leukotriene synthesis (McMillan & Walker, 1992), had no effect on des-Arg⁹-BK-induced contractions in rabbit aortic rings. Therefore, the results tend to eliminate eicosanoids, notably the contractile compounds (thromboxane A₂, PGH₂, sulphidopeptide leukotrienes) from consideration as secondary mediators of kinin action.

Two types of mechanical responses have been reported following B₁ kinin receptor stimulation in rabbit isolated blood vessels: a sustained contraction, as in the aorta (Regoli *et al.*, 1977; present results) and the mesenteric vein (Regoli

et al., 1978), and a relaxation, sometimes preceded by a brief contraction, in the mesenteric and coeliac arteries (Churchill & Ward, 1986; Ritter *et al.*, 1989). The relaxations are not prevented by removing the endothelium in the latter tissues (deBlois & Marceau, 1987; Ritter *et al.*, 1989), but the relaxations can be reversed to a B₁-receptor-mediated contraction in the presence of indomethacin. These observations suggest that the contractile events produced by B₁ receptor stimulation are masked by prostaglandin-induced relaxations in the mesenteric and coeliac arteries. The contractile effect of des-Arg⁹-BK in the rabbit aorta and mesenteric vein is resistant to many pharmacological antagonists (Regoli *et al.*, 1977; 1978) and is apparently not dependent on secondary released mediators. We provide evidence here for the existence of increased PGI₂ formation in the aortic preparation stimulated with des-Arg⁹-BK (Table 2). However, the inhibition of this release by indomethacin did not modify the mechanical response of the treated tissues (Table 1). It is possible that the increased PGI₂ production is a uniform response of large rabbit vessels to B₁ receptor stimulation, but that the local sensitivity to prostanoids varies from one preparation to another. Indeed, the rabbit aorta does not exhibit a relaxant response to PGI₂ or PGE₂, the major eicosanoids produced by rabbit isolated vessels under basal or stimulated conditions (Förstermann *et al.*, 1984). On the contrary, the mesenteric and coeliac arteries are relaxed by low concentrations of these prostaglandins (Simmet & Hertting, 1980; Förstermann *et al.*, 1984; Demirel & Türker, 1991), and their dominant mechanical relaxation response to des-Arg⁹-BK is probably dependent on prostaglandin receptor stimulation.

SMCs derived from aortic tissue without endothelium cultured over weeks retained the distinctive capacity to respond to kinins via B₁ receptors. An acute release of PGI₂ was observed in response to des-Arg⁹-BK, and the use of antag-

onists confirmed the receptor identity (Figure 4, Table 3). The phosphoinositide response to des-Arg⁹-BK was also attributed to B₁ receptors, according to the effect of antagonists selective for the B₁ and B₂ receptor types. The increased IP concentration after 5 min exposure to des-Arg⁹-BK, evidence of the activation of phospholipase C, was relatively weak under basal conditions, but was potentiated in cells pretreated with IL-1 β . This cytokine pretreatment has not been tested for PGI₂ release, because IL-1 is known to be a powerful prostaglandin release stimulus in rabbit vascular tissue (Marceau *et al.*, 1991). The activation of phospholipase C following the activation of B₁ receptors is consistent for the participation of diacylglycerol-regulated protein kinase C in the contractile effect of des-Arg⁹-BK that was proposed in the paragraphs above, in reference to the effect of protein kinase inhibitors. Significant increases in IP₃ concentrations were also recorded 5 min after stimulation with des-Arg⁹-BK in IL-1 pretreated cells (Table 4). The IP₃ (inositol 1,4,5-triphosphate)-mediated release of intracellular calcium stores in the cells could also be a distinctive feature of B₁ receptor pharmacology in blood vessels, and is also consistent with the involvement of intracellular calcium inferred from the contractility studies. Thus, at least two second messengers derived from phosphatidylinositol lipids, IP₃ and diacylglycerol, may be important in the B₁ receptor signal transduction process.

Hoe-140, at the concentration of 1.7 μ M, occasionally stimulated IP turnover in cultured SMCs, either alone or as a potentiating agent for des-Arg⁹-BK (Table 4). However, these effects did not reach statistical significance, and the phenomenon may be related to a non-specific effect at this relatively high drug concentration. The B₂ receptor antagonist, Hoe-140, consistently failed to inhibit the effects of kinins in SMCs. The pharmacological analyses based on the order of potency of agonists (des-Arg⁹-BK > BK) and on the effect of selective antagonists indicate that bradykinin B₁ receptors mediate the biochemical responses to kinins in this cell type.

The tissue source of cultured arterial SMCs apparently determines the type of kinin receptors that are expressed. In

the present study, only B₁ receptors were detected in cells derived from the rabbit aorta, but SMCs derived from the rat mesenteric artery expressed predominantly B₂ receptors (Dixon *et al.*, 1990). However, functional responses to the B₁ receptor agonist, des-Arg⁹-BK, were present at 10⁻⁷–10⁻⁶ M, with an intensity of about 20% of the maximal effect of BK in rat SMCs (Dixon *et al.*, 1990), which is suggestive of B₁ receptor co-expression. Thus, cultured SMCs derived from the rabbit aorta provide a relevant model pertinent to the investigation of the vascular effects of B₁ receptor stimulation, the release of vasorelaxant prostaglandin and direct vascular contraction. Unlike the freshly isolated rabbit aorta, but similar to aortic tissues incubated for several hours, the cultured cells retain a certain level of responsiveness to des-Arg⁹-BK after weeks of culture. The potentiation of des-Arg⁹-BK-induced phospholipase C activation by IL-1 β provides preliminary evidence for an immunological regulation of B₁ receptor function, as observed in the cardiovascular system of the rabbit (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988; 1991). The cellular model will be very useful for analysing the effect of cytokines and growth factors on the receptor population itself, and in identifying whether these factors regulate the kinin response through modifications of receptor density and/or affinity, or at the post-receptor level.

Note added in proof

While this manuscript was being processed, a report (Tropea, Gummelt, Herzig & Fredrik Leeb-Lundberg, 1993) appeared showing that des-Arg⁹-BK promotes the formation of inositol phosphate and release of arachidonic acid in cultured rabbit superior mesenteric artery smooth muscle cells.

Supported by the Medical Research Council of Canada (grant MA-9358) and the Fonds de la recherche en Santé du Québec (FRSQ). G.D. and F.M. are Scholars of the FRSQ. We thank Ms Johanne Bouthillier and Mr Claude Villeneuve for technical assistance, Mr Claude Chamberland for assistance with fluorescence microscopy and Dr Gaëtan Guillemette for advice on the technical aspects of cellular phosphoinositide measurements.

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(Received January, 28, 1993)

Revised April 7, 1993

Accepted April 8, 1993