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Regulation of kinin-induced contraction and DNA synthesis by inflammatory cytokines in the smooth muscle of the rabbit aorta

Luc Levesque, Jean-François Larrivée, Dimcho R. Bachvarov, Francis Rioux, Guy Drapeau & [1]François Marceau

Centre de recherche (Université Laval), Hôtel-Dieu de Québec, 11 Côte du Palais, Québec (Québec), Canada G1R 2J6

1 In rabbit aortic rings, the contractile response to kinins is mediated by the B_1 receptors for kinins; the response is upregulated from an initial null level in a time- and protein synthesis-dependent manner. Incubation (3 h) with human recombinant interleukin-1β (IL-1β) selectively amplified the contractile response to the B_1 receptor agonist Sar-[D-Phe^8]des-Arg^9-BK, while it did not affect the contractile effect of other agents (angiotensin II, endothelin-1, phenylephrine).

2 Oncostatin M (OSM), but not macrophage migration inhibitory factor (MIF), increased the contractile response to the B_1 receptor agonist, des-Arg^9-bradykinin (des-Arg^9-BK).

3 Cultured smooth muscle cells derived from the rabbit aorta exhibit a significant des-Arg^9-BK-induced increase in [^{3}H]-thymidine incorporation if pretreated with a cyclo-oxygenase inhibitor (diclofenac) and concomitantly treated with the cytokines IL-1 or OSM. Angiotensin II, endothelin-1 or phenylephrine, alone or in the presence of IL-1β, exerted little effect on DNA synthesis in these cells.

4 The pharmacological characterization of the mitogenic response to kinins using a set of agonist and antagonist analogues is consistent with mediation by B_1 receptors. Des-Arg^9-BK-induced DNA synthesis is suppressed by prostaglandin E_2 by a prostacyclin mimetic (iloprost), by the Ser/Thr protein kinase inhibitor, H-7, and by a tyrosine kinase inhibitor (i.e. an erbstatin analogue).

5 B_1 receptor-mediated responses and their capacity to be regulated by cytokines, are retained in rabbit aortic smooth muscle cells. Such responses could be relevant to tissue repair mechanisms and hypertrophic medial responses to injury in arteries.

Keywords: Kinin B_1 receptor; smooth muscle cells; interleukin-1; oncostatin M; epidermal growth factor

Introduction

Considerable pharmacological evidence supports the broad classification of receptors for bradykinin (BK)-related peptides into the B_1 and B_2 receptor types (Farmer & Burch, 1992; Hall, 1992; Marceau, 1995). B_1 receptors for kinins mediate the contractile effects of kinins in the rabbit isolated aorta and several other preparations. The B_1 receptor is selectively stimulated by kinin fragments of BK and Lys-BK without the COOH-terminal arginine residue, such as des-Arg^9-BK and Lys-des-Arg^9-BK, and blocked by sequence-related selective antagonists (prototype [Leu^8]des-Arg^9-BK). A distinct kinin B_1 receptor has been cloned recently from a human cDNA bank and exhibits the expected pharmacological profile (Menke *et al.*, 1994).

Several isolated smooth muscle preparations expressing B_1 receptors, such as the rabbit aorta and other arteries, exhibit the unique capacity of selectively increasing their sensitivity to kinins in a time- and protein synthesis-dependent manner from an initially null level of response (Bouthillier *et al.*, 1987; deBlois & Marceau, 1987; Pruneau & Bélichard, 1993). Interleukin (IL)-1 and epidermal growth factor (EGF) can accelerate the spontaneous upregulation process in the isolated aorta (deBlois *et al.*, 1988; 1991; 1992). This may suggest that tissue injury upregulates B_1 receptor-mediated functions via the cytokine network. Applications of this concept to vascular pathology may include the hypotensive effect of des-Arg^9-BK and of Lys-des-Arg^9-BK observed only in rabbits pretreated with lipopolysaccharide (LPS) or IL-1 (Bouthillier *et al.*, 1987; Drapeau *et al.*, 1991), the local induction of B_1 receptors following direct balloon catheter injury to the rabbit carotid artery (Pruneau *et al.*, 1994), and angiogenesis in rats following intradermal implantion of a sponge containing BK and IL-1 (Hu & Fan, 1993).

Rabbit cultured aortic smooth muscle cells appear to be a useful model for further study of the B_1 receptor because they exhibit responses to kinins (release of 6-keto-prostaglandin (PG) $F_{1\alpha}$, increase of phosphoinositide turnover) that are solely mediated by B_1 receptors (Levesque *et al.*, 1993). Moreover, IP_3 production induced by des-Arg^9-BK is enhanced when smooth muscle cells are pretreated with IL-1β or epidermal growth factor (EGF) (Levesque *et al.*, 1993; Schneck *et al.*, 1994). Based on radioligand binding assays, EGF or IL-1β upregulate the number of B_1 receptors over several hours in rabbit aortic (Schneck *et al.*, 1994; Levesque *et al.*, 1995) or mesenteric artery smooth muscle cells (Galizzi *et al.*, 1994). Phospholipase C signalling mediated by B_1 receptors has also been observed in rabbit mesenteric artery smooth muscle cells (Tropea *et al.*, 1993) and rat mesangial cells (Issandou & Darbon, 1991) and is consistent with the good preservation of the contractile response to des-Arg^9-BK in the absence of extracellular calcium (Levesque *et al.*, 1993), because IP_3 mobilizes stores of intracellular calcium.

The aim of the present study was to document the role of kinin B_1 receptors in pathological alterations of vascular smooth muscle cells, with special reference to the effect of inflammatory cytokines. Such alterations may involve not only changes in the contractility of smooth muscle, but also hyperplasia and hypertrophy of smooth muscle cells, in the context of tissue repair. We have compared the effect of IL-1 on the contractility elicited by a B_1 agonist and by other agents, and also tested whether cultured aortic smooth muscle cells retain their response to these various agents. Numerous macrophages can be found in proliferative arterial tissue following injury (Stadius *et al.*, 1994). The influence of recently characterized cytokines, oncostatin M (OSM) and macrophage migration inhibitory factor (MIF), has also been evaluated on rabbit aortic rings using the contractility assay. OSM is derived from macrophages and exerts a mitogenic effect on rabbit aortic smooth muscle cells (Grove *et al.*, 1993). MIF of pituitary origin has been implicated in the lethal outcome of en-

[1] Author for correspondence.

dotoxaemia in mice (Bernhagen *et al.*, 1993). Finally, we investigated whether naive or cytokine-treated aortic smooth muscle cells could respond to kinins by a synthesis of DNA. These studies suggest that responses to B_1 receptor stimulation are preserved in cultured smooth muscle cells.

Methods

Effect of cytokine pretreatment on aortic contractility

The thoracic aorta was isolated from New Zealand white rabbits of either sex (1.5–2 kg). The vessels were cut into rings (~4 mm diameter, 3–4 mm length) and were suspended between a metal hook and a thread loop under a tension of 2 g in 5 ml tissue baths containing oxygenated (95% O_2: 5% CO_2) Krebs solution. The composition of Krebs was (mM): NaCl 117.5, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.18, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and D-glucose 5.5. The responses of endothelium-intact rings to agents were recorded isometrically as described previously (Bouthillier *et al.*, 1987). Contractility studies pursued two separate objectives. Firstly, the specificity of the potentiating effect of IL-1β on the effect of a B_1 receptor agonist was verified, by constructing full cumulative concentration-effect curves with four agonists, in aortic rings exposed continuously or not to IL-1β (5 ng ml^{-1}) for 3 h. The selected contractile stimuli were angiotensin II, phenylephrine, endothelin-1 and the B_1 receptor agonist, Sar-[D-Phe^8]des-Arg^9-BK. Sigmoidal curves are characterized by the half-maximal effective concentration (EC_{50}) and the maximal absolute contraction amplitude (g). Secondly, to study other cytokines, cumulative concentration-effect curves for the B_1 receptor agonist, des-Arg^9-BK, were constructed after 1, 3 and 6 h of incubation. Repeated stimulation with des-Arg^9-BK was carried out in order to monitor the progressive increase of responsiveness of aortic strips to the kinin metabolite. Tissues were washed with fresh Krebs buffer between stimulations. The effect of OSM or of MIF on the spontaneous sensitization process was tested by introducing the cytokines for definite periods of the *in vitro* incubation (0–1 h, 0–3 h or 5.75–6 h, to verify whether OSM or MIF had an acute potentiating effect on the contractile response to the kinin). Vascular rings from each animal were assigned randomly to each of the treatments and to the control group, so that the statistical weight of each animal is equal in each group.

Cell culture

Several distinct lines of rabbit smooth muscle cells were established and characterized as previously described (Levesque *et al.*, 1993). Cells were cultured in Medium 199 supplemented with 10% foetal bovine serum (FBS; Gibco), antibiotics (streptomycin, 50 μg ml^{-1}, and penicillin, 50 μg ml^{-1}; Gibco). They were passaged in 75 cm^2 flasks coated with 0.2% gelatin. Cells at passages 2 to 11 were used to evaluate thymidine incorporation.

Thymidine incorporation

Experiments were performed according to Goldstein & Wall (1984) with some modifications. Briefly, cells were seeded at a density of 3×10^4 per well of 2 cm^2 in complete medium (10% FBS). After 24 h, the wells were rinsed three times with medium 199. Culture medium containing 0.4% FBS and diclofenac (500 nM), when indicated, was added to the wells. After 72 h of incubation, drugs (kinin analogues, cytokines, prostaglandins, the tyrosine kinase inhibitor and the protein kinase inhibitor) were added to the medium. The serum concentration was restored to 10% in the media of some wells for positive incorporation controls. Exactly 24 h after stimulation, 0.1 μCi of [methyl-^{3}H]-thymidine (New England Nuclear, Boston MA U.S.A.; specific activity 80.0 Ci $mmol^{-1}$) was added to each well for an additional 24 h. The wells were then rinsed three times with 1 ml of 0.9% NaCl and 150 μl of a trypsin (0.05%)-EDTA (540 μM; Gibco) solution was added to detach the cells. The cell suspension was transferred to borosilicate glass tubes (12 × 75 mm) containing 1 ml of 1% Triton X-100. One ml of 40% trichloroacetic acid (Fisher, Canada) was added to the mixture which was then centrifuged for 20 min at 2500 *g*. The pellets were suspended in Ecolite + (ICN, Canada) and their radioactivity content was determined by scintillation counting.

Drugs and reagents

The following drugs and reagents were purchased from Sigma (St-Louis MO, U.S.A.): angiotensin II, endothelin-1, PGE_2 and diclofenac. H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine) and an erbstatin analogue (methyl-2,5-dihydroxy cinnamate) were from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). Des-Arg^9-BK and bradykinin were purchased from Bachem (Torrance CA, U.S.A.). All other peptides were synthesized in our laboratories (Drapeau *et al.*, 1993). EGF, OSM and interleukin-1 receptor antagonist (IL-1RA) (human recombinant forms) were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Recombinant IL-1β (human) and MIF (both murine and human; Bernhagen *et al.*, 1993) were gifts of Dr D.E. Tracey (Upjohn Co., Kalamazoo, MI, U.S.A.) and of Dr R. Bucala (Picower Institute, Manhasset, NY, U.S.A.) respectively. The selective B_2 receptor antagonist, Hoe-140 (D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK; icatibant) (Hock *et al.*, 1991) was a gift from Dr K. Wirth, (Hoechst AG, Frankfurt, Germany), and iloprost (a PGI_2-mimetic), a gift from Berlex (Lachine, Canada). Stock solutions of drugs were made up in isotonic saline except for diclofenac (0.1 M Na_2CO_3) and erbstatin analogue (dimethylsulphoxide (DMSO) final concentration 0.1%).

Statistical analysis

Data are mean ± s.e.mean. Statistical analysis was generally made by Kruskal-Wallis test followed by Mann-Whitney test using the InStat 2.0 computer programme (GraphPad Software, San Diego, CA, U.S.A.). $P < 0.05$ was considered significant.

Results

Effect of IL-1β on aortic contractile responses to various vasoactive agents

The results of the concentration-effect curves for four vasoactive agents are shown in Table 1. The high affinity and metabolically stable agonist of the B_1 receptors, Sar-[D-Phe^8]des-Arg^9-BK, exerted a significantly greater contractile force in tissues exposed to IL-1β for 3 h. Moreover, the EC_{50} (half-maximal concentration) was significantly lower in tissues exposed to the cytokine, also affecting the potency of the kinin analogue. These findings are consistent with the reported effect of IL-1 treatment on contractions induced by the alternate B_1 receptor agonist, des-Arg^9-BK, in the same system (deBlois *et al.*, 1988). However, pretreatment with IL-1β did not influence the contractile effect of three other potent vasoactive substances, angiotensin II, phenylephrine and endothelin-1, which bind to other receptor types (Table 1).

Effect of OSM or MIF pretreatment on aortic contractility

Rabbit aortic rings were initially not responsive to the B_1 receptor agonist, des-Arg^9-BK (concentration-effect curves established after 1 h of incubation, data not shown), but the tissues spontaneously and progressively became more responsive as a function of time (contractile forces measured at 3 and 6 h, Figure 1a and 1b, respectively). OSM (up to 25 ng

ml^{-1}) had no direct contractile effect on rabbit aortic rings. However, if applied continuously during the period 0–3 h of *in vitro* incubation, it increased the contractility of the rabbit aortic rings to des-Arg^9-BK, assessed either at the 3 or 6 h time points (Figure 1; comparison of each maximal contraction amplitude with respective controls, $P < 0.05$ by repeated measure ANOVA followed by Dunnett multiple comparison test). The application of OSM for the first hour of incubation was not significantly effective in increasing the response to the kinin. OSM exerted no effect when it was administered 15 min before the stimulation with des-Arg^9-BK at the 6 h time point, indicating that the potentiating effect of OSM is not a rapid phenomenon (Figure 1b). The same data on the contractile effect of des-Arg^9-BK were also expressed as a percentage of the maximal contraction for each time point (i.e. 3 and 6 h) (Figure 1c, 1d); the EC_{50} of the B_1 agonist remained relatively constant as a function of time (150 nM, approximately), and OSM treatment had only small, non significant effects on this measurement of potency. Contractions induced by phenylephrine (500 nM) were also measured in all tissues at the time 1.5 and 6.5 h. They were relatively constant in amplitude and were not affected by OSM (data not shown).

Both human and murine MIF (up to 200 ng ml^{-1}) failed to influence significantly the contractile response to des-Arg^9-BK in rabbit aortic rings (not shown).

Interaction of cytokines with peptide mediators in the thymidine incorporation assay

In preliminary tests, diclofenac (500 nM), an inhibitor of the fatty acid cyclo-oxygenase, was found to be useful in revealing DNA synthesis induced by a nearly maximal concentration of des-Arg^9-BK (1.7 μM) in quiescent rabbit smooth muscle cells maintained in 0.4% FBS (Table 2). Cells that were not treated with diclofenac failed to incorporate significantly [^{3}H]-thymidine when stimulated with interleukin-1β (5 ng ml^{-1}) or des-Arg^9-BK, either alone or combined. When treated with the cyclo-oxygenase inhibitor, des-Arg^9-BK and IL-1β together produced significant DNA synthesis. In the following assays, diclofenac was routinely present in the incubation medium.

Quiescent rabbit aortic smooth muscle cells exhibited little DNA synthesis in response to the agonists of four different

Table 1 Effect of interleukin-1β (IL-1β) on the EC_{50} and maximal contraction amplitude of several agonists on rabbit aortic ring[a]

Agonists	*No treatment*		*IL-1β* (5 ng ml^{-1})	
	EC_{50} (nM)	*Max. contraction* (g)	EC_{50} (nM)	*Max. contraction* (g)
Sar[D-Phe^8]des-Arg^9-BK ($n=5$)	9.02 ± 1.14	1.02 ± 0.27	4.43 ± 0.75*	2.46 ± 0.52*
Angiotensin II ($n=5$)	2.57 ± 0.53	1.85 ± 0.47	3.93 ± 1.52	2.08 ± 0.67
Phenylephine ($n=6$)	142 ± 7.9	3.99 ± 0.39	407 ± 127	3.67 ± 0.34
Endothelin-1 ($n=5$)	9.11 ± 5.58	3.02 ± 0.59	9.19 ± 6.26	2.56 ± 0.41

[a]Cumulative concentration-effect curves were constructed after 3 h of *in vitro* incubation in the presence or absence of IL-1β (5 ng ml^{-1}). The number of replicates is indicated by *n*. Results from cytokine-treated tissues were compared with controls by the Mann-Whitney test. *$P < 0.05$.

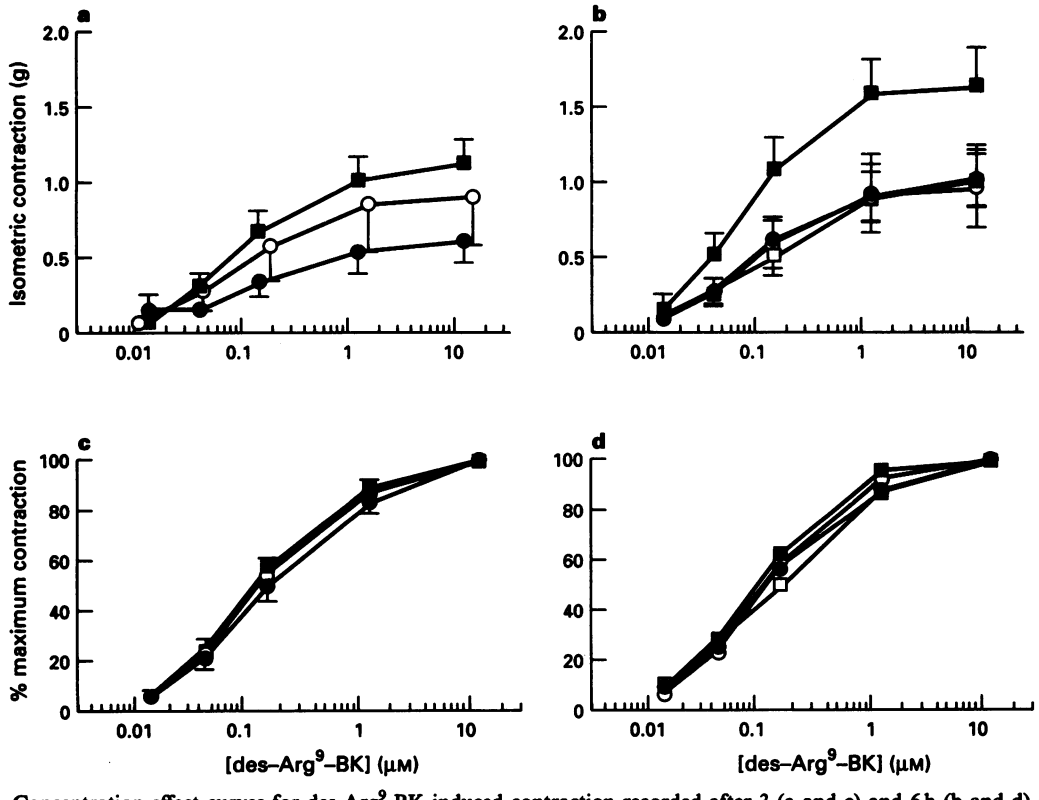

Figure 1 Concentration-effect curves for des-Arg^9-BK-induced contraction recorded after 3 (a and c) and 6 h (b and d) of *in vitro* incubation of untreated (●) rabbit aortic rings or tissues exposed to 25 ng ml^{-1} of oncostatin M during the periods (○) 0–1 h, (■) 0–3 h and (□) 5.75–6 h. Values are the mean ± s.e.mean of 7 determinations. The curves in (a) and (b) are expressed as g of developed contraction; (c) and (d) represent the same data expressed as a % of the maximal kinin-induced contraction.

receptors that were shown to mediate contraction of fresh aortic tissue (compare Figure 2 to Table 1). Serum was a positive control in the thymidine incorporation experiments (Figure 2). By contrast, IL-1 pretreatment revealed the selective effect of the B_1 receptor agonist, Sar[D-Phe^8]des-Arg^9-BK, on thymidine incorporation.

Several concentrations of cytokines that influence the contractility of B_1 receptor agonists in rabbit aortic rings were tested in the thymidine incorporation assay, to determine whether they influenced the effect of des-Arg^9-BK on DNA synthesis. Figure 3 illustrates the variation of [^{3}H]-thymidine incorporation into cells stimulated with des-Arg^9-BK (1.7 μM) and concomitantly treated with OSM, IL-1β, EGF or 10% FBS. The B_1 agonist alone does not increase thymidine incorporation in control cells or in cells treated with 10% FBS, although the latter medium promotes DNA synthesis by itself. EGF alone induced DNA synthesis in a concentration-dependent manner when compared to the appropriate basal value. Co-administration of des-Arg^9-BK and of EGF (0.3 ng ml^{-1}) significantly increased thymidine incorporation, while no supplemental effect was observed with higher EGF concentrations. IL-1β (0.025 to 5 ng ml^{-1}) does not appear to be a mitogen in rabbit aortic smooth muscle cells, based on the failure of the cytokine to increase thymidine incorporation. However, IL-1β (at and above 0.25 ng ml^{-1}) revealed a significant effect of des-Arg^9-BK in these cells. IL-1RA, a competitive antagonist of receptors for IL-1 (Dinarello, 1991), prevented the potentiating effect of IL-1β on des-Arg^9-BK-induced thymidine incorporation, suggesting that this effect of IL-1 is receptor-mediated. Low concentrations (1–10 ng ml^{-1}) of OSM did not induce DNA synthesis; however, we confirm that the larger concentration (25 ng ml^{-1}) does so in this cell type (Grove *et al.*, 1993). As found with IL-1β, OSM revealed a significant effect of des-Arg^9-BK on DNA synthesis. Thus, concentrations of 0.25 ng ml^{-1} and 2.5 ng ml^{-1} were chosen for

Table 2 [^{3}H]-thymidine incorporation induced by des-Arg^9-BK (1.7 μM), interleukin-1β (IL-1β, 2.5 ng ml^{-1}), or both stimuli combined, into rabbit aortic smooth muscle cells as a function of the presence of diclofenac (500 nM)[a]

	[^{3}H]-thymidine incorporation (c.p.m.)	
Stimulus	*Without diclofenac*	*With diclofenac*
Control	171±20	360±93
des-Arg^9-BK	202±24	283±77
IL-1β	212±30	263±49
des-Arg^9-BK + IL-1β	274±61	915±243*†

[a]Values are the mean±s.e.mean of 7 values from 2 separate experiments with different cell lines (passages 2 and 3). Kruskal-Wallis test was performed on each column (significant only in groups treated with diclofenac, $P<0.01$). The Mann-Whitney test was then applied in the diclofenac column to compare the effect of each treatment with the baseline thymidine incorporation (*$P<0.01$). The effect of the diclofenac treatment alone within each stimulus group was assessed with the Mann-Whitney test (†$P<0.01$).

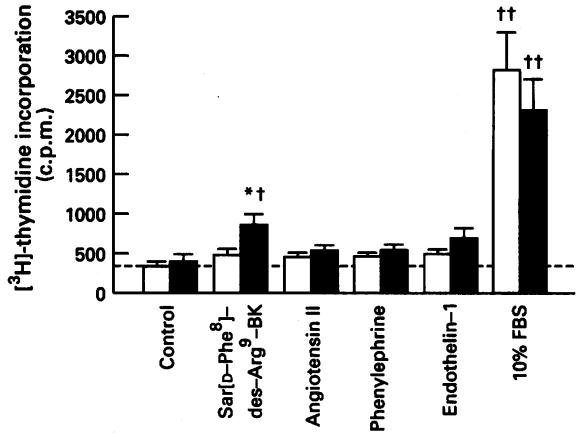

Figure 2 Effect of interleukin-1β (IL-1β, 1 ng ml^{-1}; solid columns) or of its saline vehicle (open columns) on the [^{3}H]-thymidine incorporation induced by four vasoactive agents (1 μM of each) or FBS (10%) into quiescent cultured smooth muscle cells. Values are means±s.e.mean of 16 determinations from 4 experiments that used cells at passages 3 to 6. The effect of the IL-1β treatment within each experimental group was assessed by the Mann-Whitney test; *$P<0.05$. The values were also compared to the baseline conditions (no IL-1, no vasoactive agent) using the Kruskal-Wallis test ($P<0.001$), followed by the Mann-Whitney test; †$P<0.01$; ††$P<0.001$.

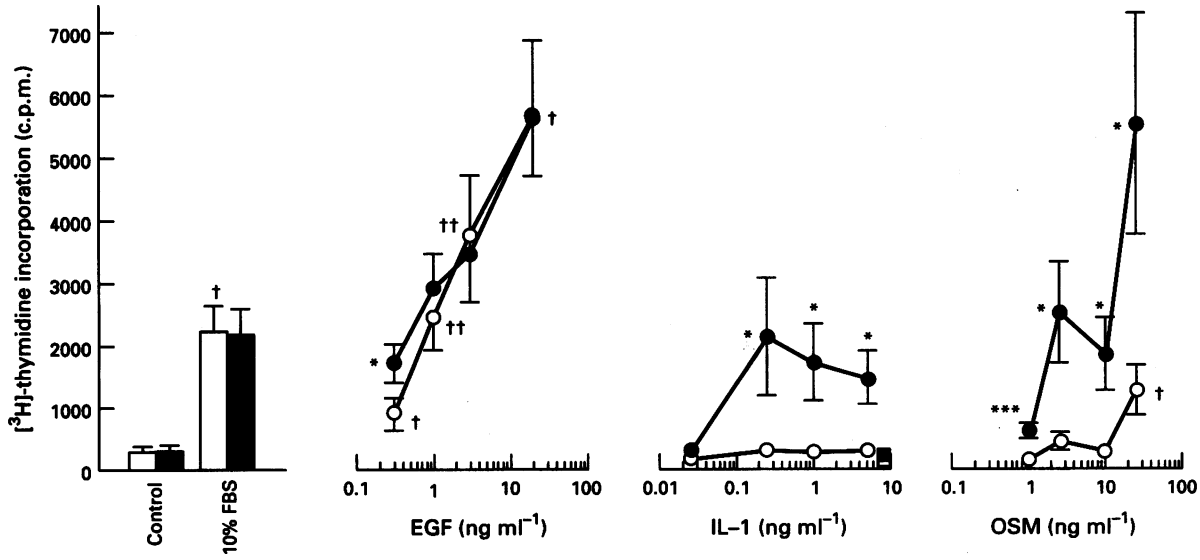

Figure 3 Effect of various treatments on [^{3}H]-thymidine incorporation induced by des-Arg^9-BK (1.7 μM; ● or solid columns) or by its saline vehicle (○ or open columns) into cultured smooth muscle cells. Values are the mean±s.e.mean of 16 to 20 determinations from 5 experiments that used cells at passages 3 to 5. The effect of des-Arg^9-BK within each treatment group was assessed by the Mann-Whitney test: *$P<0.05$, **$P<0.01$ and ***$P<0.001$. The effect of cytokine or FBS treatment on thymidine incorporation in the absence of the kinin was tested by the Kruskall-Wallis test ($P<0.001$) followed by the Mann-Whitney test: †$P<0.05$, ††$P<0.001$. IL-1RA (250 ng ml^{-1}) was co-administered with the top concentration of IL-1 (□ control; ■ with further des-Arg^9-BK treatment).

IL-1β and OSM, respectively, for further pharmacological analysis. These levels of cytokines produced optimal kinin-induced DNA synthesis when co-administered with des-Arg^9-BK, but did not affect the baseline synthesis values when administered alone.

Pharmacological characterization of kinin-induced thymidine incorporation

The pharmacological characterization of the response to kinins is shown in Figures 4 and 5. The concentration-effect relationship for kinin-induced thymidine incorporation was established for several peptides in a wide range of concentrations in the presence of either IL-1β (Figure 4a) or OSM (Figures 4b, 4c, 5). The consensus potency order of the peptides in Figure 4a and b is: Sar-[D-Phe^8]des-Arg^9-BK ≥ Lys-des-Arg^9-BK > des-Arg^9-BK > BK. No effect was observed for agonist concentrations below 1 nM. Sar-[D-Phe^8]des-Arg^9-BK is a metabolically resistant analogue of the high affinity B_1 agonist, Lys-des-Arg^9-BK (Drapeau *et al.*, 1991; 1993). The concentration-effect curve for Sar-[D-Phe^8]des-Arg^9-BK is displaced to the right by the selective B_1 receptor antagonist, Lys-[Leu^8]des-Arg^9-BK (100 nM), but not by Hoe-140 (100 nM), a selective and potent B_2 receptor antagonist (Figure 4c). These antagonists had no direct effect on baseline incorporation and only Lys-[Leu^8]des-Arg^9-BK inhibited the response induced by the alternate B_1 agonist, des-Arg^9-BK (1 μM; Figure 5).

Inhibitory drugs were used in order to characterize further the mechanisms of kinin-induced DNA synthesis (Table 3). Inhibitors had no significant direct effect on the cells stimulated with OSM (2.5 ng ml^{-1}) or with 10% FBS. Both PGE_2 and iloprost (a PGI_2 analogue) inhibited the response in cells treated with des-Arg^9-BK and OSM. H-7 (30 μM), a protein kinase inhibitor, reduced the response induced by the B_1 agonist down to the baseline value, but exerted only a marginal effect on FBS-induced DNA synthesis. The erbstatin analogue, a tyrosine kinase inhibitor, suppressed thymidine incorporation under all conditions.

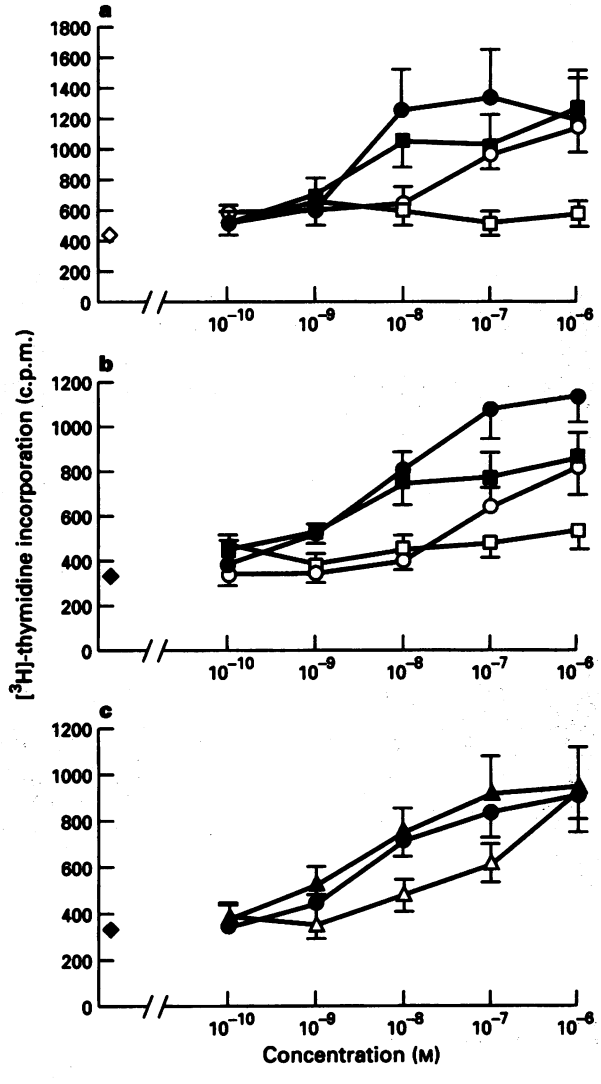

Figure 4 Concentration-effect curves for [^{3}H]-thymidine incorporation into rabbit aortic smooth muscle cells induced by des-Arg^9-BK (○), Lys-des-Arg^9-BK (■), Sar-[D-Phe^8]des-Arg^9-BK (●) and bradykinin (□) in cells treated with (a) interleukin-1β (IL-1β) 0.25 ng ml^{-1} and (b) oncostatin M (OSM) 2.5 ng ml^{-1}. (c) Dose-response curves induced by Sar-[D-Phe^8]des-Arg^9-BK alone (●) or in the presence of Hoe-140 (100 nM; ▲) or Lys-[Leu^8]des-Arg^9-BK (100 nM; △) in cells treated with OSM 2.5 ng ml^{-1}. Values are the means ± s.e.mean of 16 values from 4 experiments using cells at passages 3 to 6. The basal incorporation values (no kinins) of the IL-1 treated cells (◇) and the OSM-treated cells (◆) are the mean ± s.e.mean of 48 and 42 values, respectively, from the same cell lines. In (a) and (b) values corresponding to the effect of peptides were compared to their respective controls using the Kruskal-Wallis test ($P < 0.001$), followed by the Mann-Whitney test. Values were significantly different from the controls ($P < 0.05$) at and above 10^{-8} M (a) or 10^{-9} M (b) for both Sar-[D-Phe^8]des-Arg^9-BK and Lys-des-Arg^9-BK and at and above 10^{-8} M (a) or 10^{-7} M (b) for des-Arg^9-BK. The effect of BK was significant only in (b) at and above 10^{-7} M.

Discussion

The previously documented potentiation of des-Arg^9-BK-induced contraction by IL-1 treatment has been confirmed in the present paper, by use of the alternate agonist Sar-[D-Phe^8]des-Arg^9-BK (Table 1). Furthermore, other peptide or nonpeptide agents do not interact with IL-1 in the contractility assay. The contractility assay also shows that OSM stimulates the rate of acquisition of the response to exogenous des-Arg^9-BK in rabbit isolated aortic rings. These new results add to the evi-

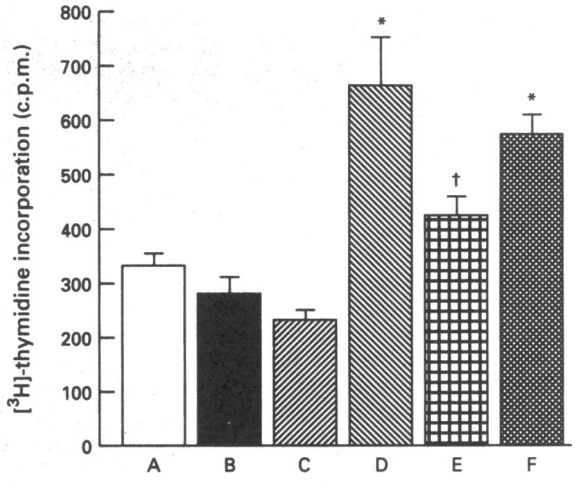

Figure 5 Pharmacological characterization of [^{3}H]-thymidine incorporation into rabbit aortic smooth muscle cells treated with oncostatin M (OSM) 2.5 ng ml^{-1}. Histograms represent basal value (A), cells treated with Lys-[Leu^8]des-Arg^9-BK 100 nM (B), Hoe-140 100 nM (C), des-Arg^9-BK 1 μM (D), des-Arg^9-BK 1 μM + Lys-[Leu^8]des-Arg^9-BK 100 nM (E) and des-Arg^9-BK 1 μM + Hoe-140 100 nM (F). Values are the mean ± s.e.mean of 16 values from 4 experiments from cell lines at passages 3 to 6. The basal value (A) is the mean ± s.e.mean of 42 values from the same cell lines. Kruskal-Wallis test indicated that the groups were not homogeneous ($P < 0.001$). Mann-Whitney test was then applied to compare the effects of each treatment with the control: *$P < 0.001$. The effect of des-Arg^9-BK without antagonist was also compared with values from cells exposed to both des-Arg^9-BK and the antagonist (Lys-[Leu^8]des-Arg^9-BK or Hoe-140) using Mann-Whitney test: †$P < 0.05$.

Table 3 Pharmacological modulation of des-Arg^9-BK-induced thymidine incorporation into rabbit aortic smooth muscle cells

Treatment	[^{3}H]-thymidine incorporation (c.p.m.) OSM (2.5 ng ml^{-1})	OSM + des-Arg^9-BK (1.7 μM)	10% FBS
None (Control)[a]	518 ± 94	1687 ± 210	2086 ± 216
PGE_2 300 nM	1003 ± 260	1155 ± 341*	2239 ± 295
Iloprost 300 nM	604 ± 189	758 ± 174**	1599 ± 286
H-7 10 μM	787 ± 221	1577 ± 189	1856 ± 348
H-7 30 μM	506 ± 50	587 ± 84***	1547 ± 197
Kruskal-Wallis test	NS	$P < 0.001$	NS
Vehicle (0.1% DMSO)[b]	804 ± 115	1373 ± 141	2440 ± 545
Erbstatin analogue 100 μg ml^{-1}	30 ± 9*	13 ± 1*	395 ± 132*

[a]Values are the mean ± s.e.mean of 12 values from 3 separate experiments with different cell lines (passages 9 and 11). Kruskal-Wallis test was performed on each column (except for the last two treatments), followed by the Mann-Whitney test using the no treatment group as a control: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.
[b]Mann-Whitney test was applied to compare the treated group (i.e. erbstatin analogue) with the appropriate control (vehicle): *$P < 0.001$.

dence of the selective cytokine-B_1 receptor interaction. OSM is a product of macrophages that binds receptors sharing the subunit gp130 with receptors for IL-6 and leukaemia inhibitory factor (Bruce *et al.*, 1992). Thus, OSM is not clearly related to other factors, such as IL-1 and EGF, that also selectively potentiate the B_1-mediated contractile responses (de Blois *et al.*, 1991; 1992). By contrast, several other cytokines fail to influence the spontaneous upregulation of aortic response to des-Arg^9-BK (deBlois *et al.*, 1989), even some that have been implicated in the complications of endotoxaemia, such as tumour necrosis factor (deBlois *et al.*, 1989) or MIF (present results). The set of cytokines that affect the process may be representative of a set of receptors expressed by rabbit aortic smooth muscle cells, without highly specific relationship to a given signalling pathway. The spontaneous increase of responsiveness to kinins in the absence of exogenous cytokines may be related to the existence of an autocrine cytokine system, as the fresh rabbit aortic tissue produces IL-1α following isolation and incubation, even under sterile conditions (Clinton *et al.*, 1991). The mitogenic action of high concentrations of OSM on rabbit aortic smooth muscle cells (Grove *et al.*, 1993) was confirmed in this report, but considerably lower levels of the cytokine potentiate kinin-induced DNA synthesis in this system.

DNA synthesis is a metabolically distant response relative to receptor stimulation, and is typically affected by many signalling pathways. Increase of DNA synthesis in response to B_1 receptor agonists in cultured aortic smooth muscle cells can be recorded only if the effect of endogenous prostanoids is inhibited by a cyclo-oxygenase blocker (e.g. diclofenac). Similar observations were previously reported, based on the human fibroblast line IMR-90, in which B_1 receptors are also linked to DNA synthesis (Goldstein & Wall, 1984). It has been documented previously that the PGI_2 metabolite, 6-keto-$PGF_{1\alpha}$, is actively released under the effect of des-Arg^9-BK in rabbit aortic smooth muscle cells (Levesque *et al.*, 1993). The PGI_2-mimetic, iloprost, and PGE_2 are suppressors of des-Arg^9-BK-induced DNA synthesis (Table 3), consistent with the postulated negative feed-back between secondarily released prostaglandins and DNA synthesis suppression. These eicosanoids are linked to adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation in vascular tissue, and a cyclic AMP analogue has previously been shown to suppress mitosis in rabbit aortic smooth muscle cells (Southgate & Newby, 1990). Within the limitations of the pharmacological approach, it can be concluded that a Ser/Thr protein kinase susceptible to H-7, like protein kinase C, is selectively involved in the thymidine incorporation induced by the B_1 receptor agonist. However, the effect of the B_1 agonist on DNA synthesis had to be revealed by a concomitant treatment with an inflammatory cytokine, particularly IL-1 or OSM, suggesting that other events were required for optimal expression of B_1 receptor function. It is interesting that serum which notably contains platelet-derived growth factor (PDGF) (Heldin & Westmark, 1990), was not suitable to reveal the effect of des-Arg^9-BK on DNA synthesis. PDGF is also one of the cytokines that fail to influence des-Arg^9-BK-induced contractions in rabbit aortic rings (deBlois *et al.*, 1989).

Substantial structure-activity information was obtained for a set of agonist and antagonist kinins in the DNA synthesis assays. This step is very important to assign properly the effects of des-Arg^9-BK to B_1 receptor stimulation. The structural correlation is excellent with data previously reported for the contractility based on the rabbit aorta (Regoli & Barabé, 1980). A minor discrepancy between the DNA and the contractility data is the similar potency of Sar-[D-Phe^8]des-Arg^9-BK relative to the natural sequence, Lys-des-Arg^9-BK, which is more active in the contractility assay and has more affinity in a binding assay (Levesque *et al.*, 1995). This is probably related to the resistance of the analogue to degradation by multiple peptidases (Drapeau *et al.*, 1993).

The present cellular model may not be fully representative of the fresh tissue, in which the upregulation of response to B_1 agonists proceeds from a null level (see above). In rabbit cultured smooth muscle cells, where some receptors are present in resting state, the abundance (B_{max}) is increased by stimulation with EGF or IL-1 (Schneck *et al.*, 1994; Galizzi *et al.*, 1994; Levesque *et al.*, 1995). OSM had a relatively modest stimulatory effect on B_1 receptor B_{max} after 24 h (Levesque *et al.*, 1995). It may be significant that the cultured cells have not retained responses to other agents that strongly stimulate contraction in the fresh normal aorta (Table 1), thus exhibiting a qualitative shift in their pharmacological response. The cytokine-induced *de novo* synthesis of B_1 receptors may represent one of the major contributing mechanisms for the obligatory role of cytokines in des-Arg^9-BK-induced DNA synthesis. However, there are probably other types of interactions between cytokines and B_1 receptor signalling. EGF, which increases significantly the receptor B_{max} after several hours of incubation, has an acute synergistic contractile effect in rabbit aortic rings (deBlois *et al.*, 1992), unlike IL-1 or OSM. The direct effect of EGF in DNA synthesis apparently obscured the effect of the B_1 receptor agonist, except for a lower concentration of EGF (Figure 3).

The function of the B_1 receptors for kinins in the rabbit aortic smooth muscle cells is subject to surprisingly complex interactions of humoral factors (cytokines, growth factors, eicosanoids) and this system could be suitable for the study of the receptor regulation. The physiological and/or pathological significance of the present results is uncertain. However, the nature of, and the mechanism underlying, the responses of vascular smooth muscle cells to agonists of the B_1 kinin re-

ceptors, and the upregulation of inflammatory cytokines, suggest that blood-derived peptides (the kinins) may interact with immunological factors (the cytokines) in the adaptative response of smooth muscle cells to injury.

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