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Studies on the induction of pharmacological responses to des-Arg^9-bradykinin *in vitro* and *in vivo*

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1 The mechanisms by which agents modulate the induction of kinin B_1-receptors were investigated by studying the effects of kinins *in vitro*, by use of the rabbit isolated aorta, and *in vivo* by measuring the blood pressure of anaesthetized rabbits.

2 The contractile response of the rabbit isolated aorta to kinins increased in a time-dependent manner *in vitro*. This effect was abolished by continuous exposure to the protein synthesis inhibitor cycloheximide (71 μM).

3 Several substances were found to increase specifically the rate of sensitization to des-Arg^9-bradykinin (des-Arg^9-Bk), when applied continuously *in vitro* to tissues isolated from normal animals: bacterial lipopolysaccharide (LPS; 1 μg ml^{-1}), muramyl-dipeptide (MDP; 2 μg ml^{-1}), phorbol myristate acetate (PMA; 320 nM), epidermal growth factor (EGF; 100 ng ml^{-1}) and endothelial cell growth factor (150 μg ml^{-1}).

4 The protease inhibitors phenylmethylsulphonyl fluoride and aprotinin, a non-adjuvant isomer of MDP, rabbit purified leukocyte interferon, fibroblast growth factor and the chemotactic peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) did not have this effect.

5 It has been demonstrated that LPS induces B_1-receptors in rabbits enabling des-Arg^9-Bk to act as a hypotensive agent. In these experiments neutropenia induced by nitrogen mustard, did not prevent the *in vivo* effect of LPS. MDP (300 μg) and PMA (100 μg) were also found to induce a state of responsiveness to des-Arg^9-Bk *in vivo*. FMLP (1 mg i.v.) induced a temporary decrease in blood neutrophil counts but had no effect on the induction of responses to des-Arg^9-Bk.

6 The development of responses mediated by the B_1-receptor in the two experimental systems seems to be unrelated to the activation of neutrophil leukocytes, but may be related to the activation of tissue macrophages. Approximately 3% of cultured adherent cells derived from rabbit aorta strips following protease digestion were stained for non-specific esterase, supporting such a possibility.

Introduction

Structure-activity studies in the field of bradykinin (Bk)-related peptides have led to the discovery of a subset of pharmacological preparations selectively sensitive to kinin fragments without the C-terminal arginine residue, e.g. des-Arg^9-Bk and des-Arg^{10}-kallidin (reviewed by Regoli & Barabé, 1980). The rabbit aortic strip is representative of these preparations. The development of selective and competitive antagonists for the actions of kinins in these systems (prototype Leu^8, des-Arg^9-Bk) provided additional evidence for the existence of a distinct receptor type, termed B_1, for these kinin fragments. An additional consistent behaviour of smooth muscle preparations sensitive to des-Arg^9-Bk is an increase in the response, from an initial null level, as a function of incubation time *in vitro*. This phenomenon was observed by different groups of investigators on rabbit vascular tissues (Regoli *et al.*, 1978; Whalley *et al.*, 1983), strips of human colon (Couture *et al.*, 1981), and the rat isolated duodenum (Boschcov *et al.*, 1984) and was demonstrated to be dependent on protein synthesis (Regoli *et al.*, 1978; Whalley *et al.*, 1983). Moreover, bacterial lipopolysaccharide (LPS, endotoxin) pretreatment *in vivo* induces a state of responsiveness to des-Arg^9-Bk in the cardiovascular system of the rabbit (Regoli *et al.*, 1981; Marceau *et al.*, 1984).

The present experiments have been undertaken to characterize further the mechanism of induction of vascular responses to des-Arg^9-Bk and to understand better the significance of the apparent up-regulated

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responses to des-Arg-kinins following tissue incubation *in vitro* or endotoxin injection *in vivo*. Indirect evidence is presented here that immunologically competent cells are involved in both systems.

Methods

Isolated blood vessels

Segments of thoracic aortae were removed from New Zealand white rabbits of either sex (1.0–2.5 kg) killed by stunning and exsanguination. Fat and excess connective tissue were excised and helical strips were cut according to Furchgott & Bhadrakom (1953). Tissues were tied at both ends and suspended in 5 ml organ baths containing oxygenated (95% O_2 and 5% CO_2) and warmed (37°C) Krebs solution (Marceau & Hugli, 1984). Rabbit aorta strips were 15–20 mm long and 3–4 mm wide and subjected to a baseline tension of 2 g. Changes in tension resulting from applications of pharmacologically active substances were recorded by isometric transducers (model 52-9545, Harvard Bioscience, South Natick, MA) on a Harvard chart recorder (50-9315).

Tissues were allowed to equilibrate for 1 h. The level of response to des-Arg^9-Bk was measured in most experiments by applying a near maximal concentration of the peptide (1.7 μM, ED_{95} approximately, Regoli & Barabé, 1980) and recording the contraction which plateaued in the following minutes. The stimulant was then washed away. This procedure was applied 1, 3 and 6 h after the beginning of incubation *in vitro*. In order to monitor the evolution of the response to other contractile agents in the same organs, responses to noradrenaline (NA, 100 nM) and, in some cases, to histamine (11 μM) were recorded at 1.5 and 6.5 h. Contractions are expressed in g. Several substances were introduced in tissue baths to modify the development of responses to des-Arg^9-Bk; they were applied throughout the *in vitro* incubation period. In order to minimize the effect of inter-animal variation, treated tissues were matched with paired controls from the same rabbit and results were analysed by use of Student's *t* test for paired samples.

In some experiments, the endothelial cell lining of rabbit aortic strips was removed by gently scrubbing the intimal surface with filter paper; the loss of acetylcholine (ACh)-induced relaxations in those preparations was monitored as an indication of a successful procedure, since endothelial cells are necessary for this response (Furchgott, 1983).

Blood pressure recording in anaesthetized rabbits

Rabbits (1.5–2.5 kg) were anaesthetized with sodium pentobarbitone and lidocaine and ventilated as described previously (Regoli *et al.*, 1981). A PE-90 polyethylene catheter was introduced in the left carotid artery and was used for blood pressure recording (EM751 pressure transducer, Elcomatic Ltd., Glasgow U.K.) and inta-arterial injections of Bk and des-Arg^9-Bk dissolved in saline (100 μl boluses followed by 200–300 μl saline). The animals were usually pretreated 5 h before anaesthesia with a single intravenous injection of a test substance. Blood samples were obtained from small ear arteries before, 10 min and 4 h after the injection of test substances to monitor white blood cell changes as described by Marceau *et al.* (1984). The results of blood cell counts are expressed as the absolute number of neutrophils and lymphocytes per mm^3; other leukocyte types are infrequent in peripheral rabbit blood (Weisbroth, 1974). A group of rabbits was rendered neutropenic by a single dose of nitrogen mustard (Mustargen, Merck Sharp & Dohme, Kirkland, Canada; 1.7 mg kg^{-1}; Taylor *et al.*, 1985). A blood sample for white cell count was obtained before the nitrogen mustard injection and 3 days later, before any other procedure, to check that neutropenia had been induced.

Fragments of rabbit aortic strips prepared *in vitro* as described above were digested for 3 h in a mixture of collagenase, dispase and DNAse (Koch *et al.*, 1986). The mixture was filtered over a sterile steel sieve and the resulting cell suspension concentrated by centrifugation and seeded in tissue culture wells. The culture medium was RPMI-1640 supplemented with 10% foetal bovine serum and antibiotics. After 24 h of culture, the adherent cells were stained for non-specific esterase as described previously (Yam *et al.*, 1971).

Drugs

The following drugs and compounds were purchased from Sigma (St. Louis, MO): (−)-noradrenaline, histamine dihydrochloride, acetylcholine chloride, cycloheximide, bradykinin (Bk), lipopolysaccharide (from *E. Coli* 0111:B4, product L3012), *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP), *N*-acetylmuramyl-L-alanyl-L-isoglutamine (MDP-isomer), *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), phenylmethylsulphonyl fluoride (PMSF), aprotinin, phorbol 12-myristate 13-acetate (PMA), purified rabbit leukocyte interferon, epidermal growth factor (EGF, from mouse submaxillary glands, receptor grade), fibroblast growth factor (FGF, from bovine pituitary glands). Des-Arg^9-Bk was purchased from Bachem (Torrance CA). Endothelial cell growth factor (ECGF, from bovine brain, culture grade) was from Meloy Labs., Springfield, VA. Drugs were dissolved in pyrogen-free 0.9% saline except for FMLP which was dissolved first in 0.1 M Na_2CO_3. For *in vitro* experiments, drugs were injected in small volumes (5–100 μl) into the tissue

baths at the appropriate time and concentrations are expressed at the tissue level.

Results

Rabbit aortic strips

The rabbit aortic preparation did not respond to des-Arg^9-Bk (1.7 μM) or to Bk (12 μM) after one hour of equilibration *in vitro*, but sustained and significant contractile responses were recorded at 3 and 6 h of incubation (Figure 1). Cycloheximide (71 μM), a protein synthesis inhibitor, almost completely prevented the development of the contractile response to des-Arg^9-Bk when applied continuously (Table 1). Contractile responses to noradrenaline (NA, 100 nM, Figure 1) or to histamine (11 μM, not shown) also increased as a function of incubation time but this sensitization was different from the increasing responses to des-Arg^9-Bk: it was relatively modest, unaffected by cycloheximide and the initial level of response was high.

A number of mechanisms might be involved in the induction of responses to des-Arg^9-Bk in isolated smooth muscle. Several hypotheses were examined using aortic strips isolated from normal, untreated rabbits (Table 1). Intimal damage sufficient to abolish the relaxant response to acetylcholine (100 nM) did not modify the process of *in vitro* sensitization to des-Arg^9-Bk (1.7 μM); in fact the level of response to the octapeptide was significantly greater at 3 h of incubation as compared to paired strips. In the large sample constituted by most control tissues ($n = 119$), the relaxant response to acetylcholine recorded at 6.5 h did not correlate with the contractile response to des-Arg^9-Bk recorded at 6 h ($r = -0.06$), suggesting that the variable degree of endothelial damage in control tissues is not linked to the development of the response to the Bk fragment.

The possible involvement of protease released from damaged tissue was also examined. The serine protease inhibitor PMSF (2 mM), as well as the more selective enzyme inhibitor aprotinin (60 ku ml^{-1}) did not influence the rate of sensitization to des-Arg^9-Bk. PMA selectively modified the rate: continuous exposure to PMA (200 ng ml^{-1}) slightly increased the late response (6 h) to des-Arg^9-Bk.

In a previous study, *E. coli* lipopolysaccharide (LPS) has been shown to induce a state of cardiovas-

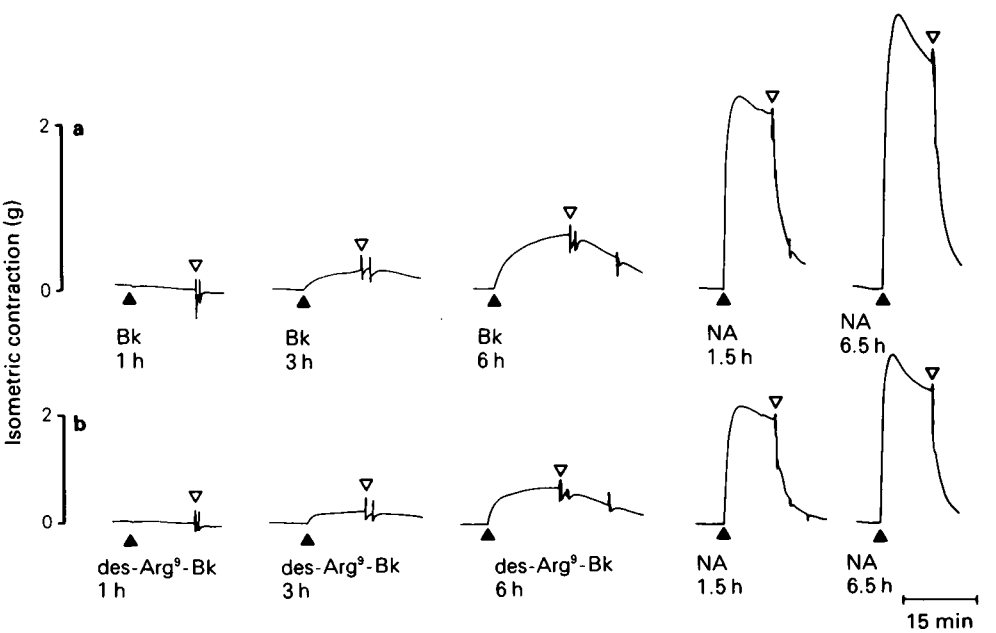

Figure 1 Contractile effect of bradykinin (Bk), des-Arg^9-Bk and noradrenaline (NA) on the rabbit aortic strip as a function of incubation time *in vitro*. Two strips of the same aorta isolated from a normal rabbit were used. In (b), des-Arg^9-Bk (1.7 μM) was applied at 1, 3 and 6 h and NA (100 nM) at 1.5 and 6.5 h. In (a), Bk (12 μM) was used to show that the up-regulated responses to the Bk fragment were also observed with Bk, although higher concentrations of the peptide were required to obtain an equivalent contraction. Abscissa scale: time; ordinate scales: isometric contraction. Closed symbols refer to the application of agents and open symbols to washout of stimulants.

Table 1 Effect of various *in vitro* treatments on the development of contractile responses to des-Arg^9-bradykinin in rabbit aortic strips

	Response to des-Arg^9-Bk			
	Treated tissues		*Paired controls*	
	3 h	*6 h*	*3 h*	*6 h*
Treatment (concentration)				
Cycloheximide (71 μM)	0 (8)**[a]	0.03 ± 0.03 (8)**	0.51 ± 0.13 (8)	1.08 ± 0.25 (8)
Intimal damage	1.22 ± 0.28 (7)*	1.73 ± 0.31 (7)	0.79 ± 0.20 (7)	1.36 ± 0.23 (7)
PMSF (2 mM)	0.27 ± 0.07 (7)	0.71 ± 0.13 (7)	0.37 ± 0.07 (7)	1.03 ± 0.10 (7)
Aprotinin (60 ku ml^{-1})	0.41 ± 0.19 (7)	0.91 ± 0.25 (7)	0.38 ± 0.11 (7)	0.90 ± 0.14 (7)
PMA (320 nM)	0.29 ± 0.07 (8)	1.06 ± 0.20 (8)*	0.29 ± 0.09 (8)	0.72 ± 0.13 (8)
LPS (1 μg ml^{-1})	0.61 ± 0.11 (8)**	1.50 ± 0.18 (8)**	0.31 ± 0.08 (8)	0.71 ± 0.10 (8)
LPS (200 ng ml^{-1})	0.30 ± 0.07 (8)	0.62 ± 0.14 (8)	0.23 ± 0.04 (8)	0.52 ± 0.10 (8)
MDP (2 μg ml^{-1})	0.43 ± 0.11 (8)**	1.00 ± 0.19 (8)**	0.25 ± 0.08 (8)	0.65 ± 0.19 (8)
MDP-isomer (2 μg ml^{-1})	0.25 ± 0.07 (7)	0.68 ± 0.15 (7)	0.34 ± 0.17 (7)	0.76 ± 0.31 (7)
Interferon (280 u ml^{-1})	0.33 ± 0.07 (8)	0.68 ± 0.13 (8)	0.41 ± 0.13 (8)	0.67 ± 0.18 (8)
ECGF (150 μg ml^{-1})	1.58 ± 0.30 (7)**	2.72 ± 0.39 (7)**	0.62 ± 0.17 (7)	1.12 ± 0.25 (7)
EGF (100 ng ml^{-1})	1.94 ± 0.20 (9)**	2.67 ± 0.37 (9)**	0.51 ± 0.06 (9)	1.08 ± 0.13 (9)
FGF (20 ng ml^{-1})	0.64 ± 0.13 (7)	1.38 ± 0.17 (7)	0.78 ± 0.18 (7)	1.48 ± 0.25 (7)
FMLP (100 nM)	0.31 ± 0.07 (7)	0.70 ± 0.13 (7)	0.36 ± 0.08 (7)	0.88 ± 0.19 (7)

[a] Contractile effect (g) of des-Arg^9-Bk (1.7 μM) recorded at the time indicated on pairs of rabbit aortic strips, one treated and one control. Statistically significant differences between the response of tested tissues and the paired controls have been evaluated by use of Student's *t* test for paired samples (*$P < 0.05$, **$P < 0.01$). Tissues were also exposed to des-Arg^9-Bk at 1 h of incubation and none of them responded at that time in any group. Responses to noradrenaline (NA; 100 nM) were evaluated in the same sets of tissues at 1.5 and 6.5 h of incubation. NA-induced contractions obtained in treated tissues were not significantly different from those measured in the paired controls, except for a depressed late response to NA in tissues with intimal damage (not shown). Abbreviations used: PMSF, phenylmethylsulphonyl fluoride; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; MDP, muramyl dipeptide; ECGF, endothelial cell growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor.

cular responsiveness to des-Arg^9-Bk in the rabbit *in vivo*; moreover, aortic strips isolated from LPS-treated animals showed a definite contractile response to des-Arg^9-Bk from the beginning of the *in vitro* incubation, suggesting that the tissue had been sensitized *in vivo* by this treatment (Regoli *et al.*, 1981). In the present set of experiments, LPS was introduced into the organ baths containing aortic strips isolated from normal animals, in order to monitor any changes in the spontaneous rate of sensitization to des-Arg^9-Bk. At the high concentration of 1 μg ml^{-1}, LPS increased selectively the contractile responses to des-Arg^9-Bk recorded at 3 and 6 h. At a lower concentration (200 ng ml^{-1}) it was ineffective. Among the numerous effects of LPS in biological systems, secretory functions of macrophages are possibly related to this action. Other known stimulants of these cells include the adjuvant peptidoglycan muramyl-dipeptide (MDP) (Chedid & Lederer, 1978). MDP (2 μg ml^{-1}) increased selectively the response to des-Arg^9-Bk at 3 and 6 h of incubation *in vitro*. The isomer of MDP, which is not an immunostimulator (Chedid & Lederer, 1978), had no effect on the rate of sensitization to des-Arg^9-Bk. Purified rabbit leukocyte interferon (280 u ml^{-1}) did not affect responses of the rabbit aorta to contractile stimuli.

Some commercially available growth factors were applied continuously *in vitro* to rabbit aortic strips at concentrations shown to be mitogenic in cell culture systems. Endothelial cell growth factor (ECGF) and epidermal growth factor (EGF), but not fibroblast growth factor (FGF), were selective and very potent stimulants of des-Arg^9-Bk-induced contractions at 3 and 6 h. None of the agents capable of stimulating the rate of sensitization to des-Arg^9-Bk induced a state of sensitivity to this peptide at 1 h of incubation, indicating a minimal lag time necessary for a response to be expressed to the octapeptide in tissues isolated from normal animals. In addition, the various treatments applied did not modify the contractile responses to NA or to histamine, as compared to the ones recorded in paired aortic strips, except for the significantly depressed late response to NA in tissues with intimal damage (not shown).

The concentration-effect curves to des-Arg^9-Bk and to Bk were established in rabbit aortic strips incubated for 4.5 to 6 h *in vitro*, in order to characterize the effect of representative stimulants, identified in Table 1, on

the biological effects of kinins (Figure 2). The concentration-effect curves, expressed as a % of maximal response, were shifted to the left in the presence of EGF, but not of LPS or MDP.

Hypotensive effect of kinins in anaesthetized rabbits

In control animals, intra-arterial bolus injections of des-Arg^9-Bk (0.25–2.5 μg) had virtually no effect on the mean arterial pressure (Table 2). Bk was a potent hypotensive agent: in control rabbits, 25 ng produced a fall in blood pressure of 10 ± 3 mmHg, 100 ng, 24 ± 4 mmHg and 250 ng, 30 ± 3 mmHg ($n = 10$). However, in animals pretreated with LPS (10 μg i.v. 5 h before anaesthesia), exogenous des-Arg^9-Bk became also a hypotensive agent, as shown previously (Regoli *et al.*, 1981; Marceau *et al.*, 1984). Bk-induced responses were unaffected by LPS treatment, or by any other treatment applied *in vivo* (not shown), suggesting a stable effect mediated by the classical (B_2) receptor type.

Animals depleted of neutrophil leukocytes by an injection of nitrogen mustard 3 days before the experimental procedure behaved in a similar manner to normal animals: they were insensitive to des-Arg^9-Bk, but became responsive if injected with LPS 5 h before the assay. This suggests that neutrophils are not involved in the sensitization process. MDP

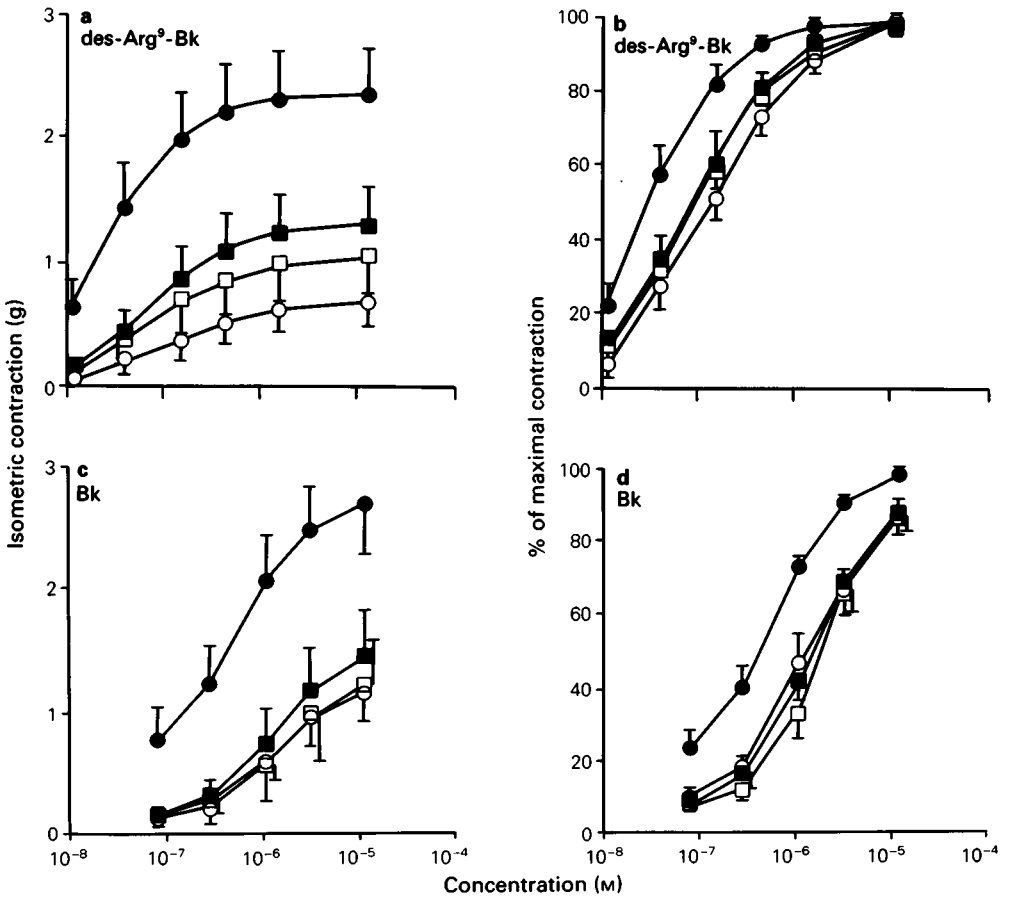

Figure 2 Concentration-effect curves for bradykinin (c and d) (Bk) and des-Arg^9-Bk (a and b) on (○) control rabbit aortic strips and on tissues continuously exposed to (●) epidermal growth factor (EGF; 100 ng ml^{-1}), (□) lipopolysaccharide (LPS; 1 μg ml^{-1}), or (■) muramyl dipeptide (MDP; 2 μg ml^{-1}). In tissues stimulated with Bk, the maximal effect was verifed by applying a supramaximal concentration of des-Arg^9-Bk. The points represent the mean of 7–8 determinations and vertical lines show s.e.mean. The curves presented in (a and c) are expressed as g of developed contraction; (b and d) represent the same data expressed as a % of the maximal kinin-induced contraction.

Table 2 Effect of des-Arg^9-bradykinin on the mean arterial blood pressure of anaesthetized rabbits subjected to various treatments *in vivo*

Treatment[a]	n	*Basal blood pressure (mmHg)*		*Hypotensive effect of des-Arg^9-Bk*		
		Systolic	*Diastolic*	*0.25 μg*	*1.0 μg*	*2.5 μg*
Controls	10	106 ± 2	74 ± 3	0[b]	0	0
LPS (10 μg)	10	104 ± 4	71 ± 4	7 ± 1***	12 ± 2***	18 ± 2***
Nitrogen mustard	6	106 ± 5	80 ± 6	0	0	0
Nitrogen mustard + LPS (10 μg)	7	100 ± 5	71 ± 4	6 ± 2**	11 ± 2***	13 ± 5***
MDP (300 μg)	10	98 ± 4	75 ± 3	1 ± 1*	4 ± 1***	7 ± 1***
MDP-isomer (300 μg)	7	111 ± 4	83 ± 3*	0	0	1 ± 1
FMLP (1 mg)	6	110 ± 3	83 ± 2*	0	1 ± 1	2 ± 1
PMA (100 μg)	8	88 ± 6**	61 ± 6*	1 ± 1	2 ± 1*	4 ± 1**
EGF (5 μg)	5	105 ± 7	69 ± 12	0	0	0

[a] Treatments were in the form of a single i.v. injection 5 h before anaesthesia, except for nitrogen mustard, injected 3 days before the experimental procedure.

[b] Fall of mean arterial blood pressure, mmHg. Results are compared with the control group by use of Student's *t* test. Significance levels are expressed as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Results obtained in animals treated with both nitrogen mustard and LPS are not significantly different from those recorded following treatment with LPS alone. For key to abbreviations used see Table 1.

(300 μg i.v.) was able to induce hypotensive responses to exogenous des-Arg^9-Bk but the magnitude of the responses was significantly less than those elicited after LPS pretreatment; higher doses of MDP did not increase the amplitude of the hypotensive responses to des-Arg^9-Bk (not shown). MDP-isomer was unable to induce hypotensive responses at a dose of 300 μg; this substance might increase slightly baseline diastolic blood pressure.

The chemotactic peptide FMLP (1 mg i.v.) did not induce a state of responsiveness to des-Arg^9-Bk *in vivo*. Pretreatment with this substance increased baseline diastolic blood pressure as compared to control animals.

PMA (100 μg i.v.) induced small but significant *in vivo* hypotensive responses to exogenous des-Arg^9-Bk (1.0 and 2.5 μg). Higher doses of PMA were frequently lethal for rabbits. The dose used in the present study decreased both baseline systolic and diastolic blood pressure. Epidermal growth factor (EGF, 5 μg i.v.) did not induce a state of responsiveness to des-Arg^9-Bk.

In order to show the possible synergism between PMA and LPS or EGF and LPS, these substances were combined at the same doses used in the experiments described above. Simultaneous pretreatment with PMA and LPS or with EGF and LPS induced cardiovascular responses to des-Arg^9-Bk not significantly different from the ones recorded in LPS pretreated animals (not shown).

White blood cell counts were obtained systematically from the animals used in the blood pressure experiments described above (Table 3). LPS induced in 10 min a slowly reversible neutropenia and a progressive decline in the lymphocyte counts. The most constant effect of MDP was a relative lymphopenia 4 h after injection. MDP-isomer had no consistent effect on the white blood cell counts except for a short term decrease in the lymphocyte counts. FMLP had a remarkable effect on the neutrophil counts: a sudden drop 10 min after injection was followed by a rebound. The lymphocyte counts were little affected by FMLP; it decreased them only transiently. PMA elicited a sudden neutropenia 10 min after injection followed by a return to the initial level, as determined 4 h later; a slowly reversible lymphopenia was also produced by PMA. EGF did not alter the white blood cell counts consistently.

Approximately 3% of cultured adherent cells derived from the rabbit aorta following protease digestion were strongly positive for non-specific esterase, indicating the presence of a significant number of cells of the monocyte-macrophage lineage in this tissue.

Discussion

In the present study, several treatments were applied to two assays for des-Arg^9-Bk: the isolated rabbit aorta, where a progressive increased responsiveness to the octapeptide was seen, and rabbit blood pressure.

The rabbit aortic strip behaved much like the mesenteric vein and the basilar artery isolated from the same animal, since a protein synthesis inhibitor, cycloheximide, prevented the expression of responses to des-Arg^9-Bk without modifying the actions of other

Table 3 White blood cell counts per mm³ in animals used to obtain data presented in Table 2

Treatment[a]	n	Neutrophil counts			Lymphocyte counts		
		Before injection	*10 min after injection*	*4 h after injection*	*Before injection*	*10 min after injection*	*4 h after injection*
Controls	11	3130 ± 517[b]			5657 ± 593[b]		
LPS (10 μg)	6–13	2503 ± 335	332 ± 143***[c]	512 ± 118***	5415 ± 1785	3847 ± 546	1152 ± 256**
Nitrogen mustard	7	3011 ± 672	61 ± 22[d]***		4632 ± 510	1193 ± 222[d]***	
Nitrogen mustard + LPS (10 μg)	8	3236 ± 478	27 ± 16[d]***		5592 ± 648	1577 ± 390[d]***	
MDP (300 μg)	7–11	3059 ± 297	2825 ± 612	6956 ± 1599	4992 ± 943	3340 ± 645	1903 ± 336**
MDP-isomer (300 μg)	9	3767 ± 736	2643 ± 271	2881 ± 267	5874 ± 559	4221 ± 451*	4737 ± 517
FMLP (1 mg)	8	3431 ± 534	66 ± 30***	6034 ± 1240	4818 ± 692	1809 ± 385**	3147 ± 570
PMA (100 μg)	9–11	4121 ± 576	70 ± 43***	3331 ± 526	5339 ± 683	1236 ± 364***	3504 ± 450*
EGF (5 μg)	6	4295 ± 956	2822 ± 735	4997 ± 1589	4622 ± 954	3595 ± 773	5987 ± 1154

[a] Treatments were in the form of a single i.v. injection 5 h before anaesthesia, except for nitrogen mustard, injected 3 days before the experimental procedure.
[b] Blood from control animals was sampled one hour before anaesthesia.
[c] Each value is compared with pretreatment value by use of Student's *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[d] Blood from nitrogen mustard-treated animals was sampled before and 3 days after the injection of the anti-mitotic drug. The second sampling took place before LPS injection in rabbits subjected to both treatments. For key to abbreviations used see Table 1.

myotropic agents (Regoli *et al.*, 1978; Whalley *et al.*, 1983). The protease inhibitors PMSF and aprotinin did not modify the acquisition of responses to des-Arg⁹-Bk by the rabbit aortic strip, indicating that enzymes susceptible to these inhibitors are not involved in the process. Intimal damage, sufficient to abolish ACh-induced relaxant responses of the aortic strip, increased the responses to des-Arg⁹-Bk only slightly in the present study. Thus, direct mechanical damage to tissues appears to be a secondary factor in the expression of tissue responses to des-Arg⁹-Bk.

The sensitization process in aortic strips is a spontaneous process of unknown mechanism; it is stimulated by three substances also found to induce responses to exogenous des-Arg⁹-Bk *in vivo*: LPS, MDP and PMA. A common biological property of LPS, MDP and, to a lesser extent, PMA, is the stimulation of the synthesis of certain proteins in macrophages. For instance, the expression of the surface antigen Mo3e by human monocytes is stimulated by LPS, MDP and PMA but not by FMLP or interferon (Todd *et al.*, 1985). LPS and MDP are known stimulants of interleukin 1 production in these cells (Warren *et al.*, 1986). The peptidoglycan MDP is especially selective for macrophage-like cells in short-term experimental systems (Chedid & Lederer, 1978; Warren *et al.*, 1986).

The stimulatory effect of growth factors on isolated tissues is more difficult to interpret. EGF is known to stimulate phagocytosis in macrophages (Laskin *et al.*, 1980). On the other hand, monocytes stimulated with LPS synthesize and release platelet-derived growth factor (Martinet *et al.*, 1986), suggesting the possibility that a growth factor may be the final mediator of LPS, MDP and PMA in tissues. Still other explanations of the action of EGF and ECGF are possible: they may act in synergy with kinins at the muscle cell level, affecting both the maximal effect and the ED_{50} (Figure 2), without being capable of initiating the sensitization to kinins (EGF did not induce the state of responsiveness to des-Arg⁹-Bk *in vivo* at the dose tested). More work involving pure cell populations is needed to dissect the mechanism of action of each stimulant.

In animals depleted of neutrophil leukocytes by nitrogen mustard, LPS retained its capacity to induce responses to des-Arg⁹-Bk, indicating that neutrophil leukocytes are not involved in the mechanism of induction. FMLP, a strong activator and chemotactic agent for neutrophils, was not an inducer of responses to exogenous des-Arg⁹-Bk, further supporting the absence of a relationship between induction of responses and a neutrophil action on the blood vessel walls.

The three substances identified as inducers *in vivo*, LPS, MDP and PMA, were the only ones tested that produced a significant decrease of lymphocyte counts 4 h after injection, possibly indicating a cytokine-mediated mobilization of circulating lymphocytes.

This could be taken as additional indirect evidence for the involvement of immunologically competent cells in the sensitization of vascular tissue to des-Arg^9-Bk.

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References

BOSCHCOV, P., PAIVA, A.C.M., PAIVA, T.B. & SHIMUTA, S.I. (1984). Further evidence for existence of two receptor sites for bradykinin responsible for the diphasic effect in the rat isolated duodenum. *Br. J. Pharmac.*, **83**, 591–600.

CHEDID, L. & LEDERER, E. (1978). Past, present and future of the synthetic immunoadjuvant MDP and its analogs. *Biochem. Pharmac.*, **27**, 2183–2186.

COUTURE, R., MIZRAHI, J., REGOLI, D. & DEVROEDE, G. (1981). Peptides and the human colon: an *in vitro* pharmacological study. *Can. J. Physiol. Pharmac.*, **59**, 957–964.

FURCHGOTT, R.F. (1983). Role of endothelium in responses of vascular smooth muscle. *Circulation Res.*, **53**, 557–573.

FURCHGOTT, R.F. & BHADRAKOM, S. (1953). Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs. *J. Pharmac. exp. Ther.*, **108**, 129–143.

KOCH, A.E., POLVERINI, P.J. & LEIBOVICH, S.C. (1986). Stimulation of neovasculatization by human rheumatoid synovial tissue macrophages. *Arthritis Rheum.*, **29**, 471–479.

LASKIN, D.L., LASKIN, J.D., WEINSTEIN, B. & CARCHMAN, R.A. (1980). Modulation of phagocytosis by tumor promoters and epidermal growth factor in normal and transformed macrophages. *Cancer Res.*, **40**, 1028–1035.

MARCEAU, F. & HUGLI, T.E. (1984). Effects of C3a and C5a anaphylatoxins on guinea pig isolated blood vessels. *J. Pharmac. exp. Ther.*, **230**, 749–754.

MARCEAU, F., LUSSIER, A. & ST-PIERRE, S. (1984). Selective induction of cardiovascular responses to des-Arg^9-bradykinin by bacterial endotoxin. *Pharmacology*, **29**, 70–74.

MARTINET, Y., BITTERMAN, P.B., MORNEX, J.-F., GROTENDORST, G.R., MARTIN, G.R. & CRYSTAL, R.G. (1986). Activated human monocytes express the c-*cis* proto-oncogene and release a mediator showing PDGF-like activity. *Nature*, **319**, 158–160.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmac. Rev.*, **32**, 1–46.

REGOLI, D., MARCEAU, F. & BARABÉ, J. (1978). *De novo* formation of vascular receptors for bradykinin. *Can. J. Physiol. Pharmac.*, **56**, 674–677.

REGOLI, D., MARCEAU, F. & LAVIGNE, J. (1981). Induction of the B_1-receptors for kinins in the rabbit by a bacterial lipopolysaccharide. *Eur. J. Pharmac.*, **71**, 105–115.

TAYLOR, R.G., McCALL, C.E., THRALL, R.S., WOODRUFF, R.D. & O'FLAHERTY, J.T. (1985). Histopathologic features of phorbol myristate acetate-induced lung injury. *Lab. Invest.*, **52**, 61–70.

TODD, R.F., ALVAREZ, P.A., BROTT, D.A. & LIU, D.Y. (1985). Bacterial lipopolysaccharide, phorbol myristate acetate, and muramyl dipeptide stimulate the expression of a human monocyte surface antigen Mo3e. *J. Immunol.*, **135**, 3869–3877.

WARREN, S.H., VOGEL, R.F. & CHEDID, L.A. (1986). Current status of immunological adjuvants. *A. Rev. Immunol.*, **4**, 369–388.

WEISBROTH, S.H. (1974). The Biology of the Laboratory Rabbit. New York: Academic Press.

WHALLEY, E.T., FRITZ, H. & GEIGER, R. (1983). Kinin receptors and angiotensin converting enzyme in rabbits basilar arteries. *Naunyn-Schmiedebergs Arch. Pharmac.*, **324**, 296–301.

YAM, L.T., LI, C.Y. & CROSBY, W.H. (1971). Cytochemical identification of monocytes and granulocytes. *Am. J. clin. Path.*, **55**, 283–289.

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