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Endothelium-dependent relaxations mediated by inducible B_1 and constitutive B_2 kinin receptors in the bovine isolated coronary artery

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1 Rings of bovine left anterior descending coronary artery (LAD) were contracted with the thromboxane A_2-mimetic, U46619 (1-30 nM), to approximately 40% of their maximum contraction to 125 mM KCl Krebs solution ($KPSS_{max}$) for comparison of responses to the B_1 and B_2 kinin receptor agonists, des-Arg^9-bradykinin (des-Arg^9-BK) and bradykinin (BK), respectively. Relaxation responses were normalized as percentages of the initial U46619-induced contraction level, while contractile responses were expressed as percentages of $KPSS_{max}$.

2 After 6 h of *in vitro* incubation in Krebs solution at 37°C, des-Arg^9-BK (pEC_{50}, 8.00±0.08; maximum response (R_{max}), 93.9±1.9%) and BK (pEC_{50}, 9.75±0.07; R_{max}, 100.1±0.7%) caused endothelium-dependent relaxations in precontracted rings of bovine LAD which were competitively and selectively antagonized by the B_1 receptor antagonist, des-Arg^9-[Leu^8]-BK (pA_2, 6.27±0.11) and the B_2 receptor antagonist Hoe-140 (pA_2, 9.63±0.14), respectively.

3 At 3 h of *in vitro* incubation, the sensitivity (pEC_{50}, 7.45±0.10) and R_{max} (84.6±3.3%) to des-Arg^9-BK were significantly less than those obtained in the same tissues at 6 h (pEC_{50}, 7.94±0.06; R_{max}, 91.4±2.5%), whereas endothelium-dependent relaxations to BK and ACh were unaffected by incubation time.

4 Relaxation responses to des-Arg^9-BK, but not BK, at both 3 h and 6 h were significantly attenuated by the protein synthesis inhibitors, cycloheximide (30 and 100 μM) and actinomycin D (2 μM).

5 At 6 h, the nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine (L-NOARG, 100 μM), caused a significant 2 fold decrease in pEC_{50} (9.58±0.03) but had no effect on R_{max} for BK. For des-Arg^9-BK, L-NOARG (100 μM) caused a marked and significant decrease in both the pEC_{50} and R_{max} and revealed contractions to low concentrations of des-Arg^9-BK. In both cases, L-NOARG inhibition was reversed in the presence of L-arginine (10 mM).

6 At 6 h, removal of the endothelium abolished relaxation responses to des-Arg^9-BK and BK, and for des-Arg^9-BK, but not BK, unmasked concentration-dependent contractions (pEC_{50}, 7.57±0.09; R_{max}, 83.4±9.1%). The sensitivity of contractions to des-Arg^9-BK increased slightly from 3 h (pEC_{50}, 7.37±0.08) to 6 h (pEC_{50}, 7.62±0.12) of *in vitro* incubation; however, there was a small but significant depression in the maximum response over this time (R_{max}, 126.8±8.5% and 103.3±8.6% for 3 h and 6 h of incubation respectively).

7 In conclusion, the bovine LAD contains inducible B_1 and constitutive B_2 endothelial cell kinin receptors, both of which mediate endothelium-dependent relaxation partly via the release of NO. B_1 receptors were also present on the smooth muscle layer of the bovine LAD.

Keywords: Bradykinin; des-Arg^9-bradykinin; B_1, B_2 kinin receptors; des-Arg^9-[Leu^8]-bradykinin; Hoe-140; endothelium; cycloheximide; actinomycin D; nitric oxide; bovine coronary artery

Introduction

Kinins exert their vascular effects through either B_1 or B_2 receptors (Regoli & Barabé, 1980). B_2 receptors are classified according to their sensitivity to stimulation by the naturally occurring kinins bradykinin (BK) and kallidin (KD; Regoli & Barabé, 1980) and to inhibition by the peptide antagonist Hoe-140 (Hock *et al.*, 1991). B_1 receptors, however, are relatively insensitive to BK and KD and require the conversion of these compounds into fragments lacking their C-terminal arginine residue (eg. des-Arg^9-BK and des-Arg^{10}-KD) for activation (Regoli & Barabé, 1980). Moreover, B_1 receptors are insensitive to inhibition by Hoe-140 (Hock *et al.*, 1991), yet are competitively blocked by the kinin fragments des-Arg^9-[Leu^8]-BK and des-Arg^{10}-[Leu^9]-KD (Regoli *et al.*, 1977; Churchill & Ward, 1986). Expression cloning of both B_1 (Menke *et al.*, 1994) and B_2 (Eggerickx *et al.*, 1992; Hess *et al.*, 1993) receptors from human genomic DNA libraries and B_2 receptors from a rat uterus cell line (McEachern *et al.*, 1991) and mouse embryonic stem cells (Hess *et al.*, 1993) has confirmed the presence of both receptor types.

B_2 receptors are a constitutive component of the vasculature and mediate endothelium-dependent (D'Orleans-Juste *et al.*, 1985; Regoli *et al.*, 1986; Whalley *et al.*, 1987) and -independent (Cherry *et al.*, 1982; Regoli *et al.*, 1982) relaxations as well as direct smooth muscle contractions (Regoli *et al.*, 1986). B_1-receptor-mediated responses, however, are generally not observed under normal physiological conditions yet are induced on both endothelial and smooth muscle cells following various types of vascular trauma. Thus, induction of smooth muscle contractions (Regoli *et al.*, 1977; 1978; Deblois & Marceau, 1987; Bouthillier *et al.*, 1987) and endothelium-dependent relaxations (Deblois & Marceau, 1987; Pruneau & Belichard, 1993) to des-Arg^9-BK occurs either during incubation of isolated vessels in the organ bath or in response to balloon catheter injury *in vivo* (Pruneau *et al.*, 1994). The sensitization of vascular responses to des-Arg^9-BK has been shown to be selectively blocked by protein synthesis inhibitors

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such as cycloheximide (Regoli & Barabé, 1978; Bouthillier *et al.*, 1987; Deblois & Marceau, 1987; Pruneau & Belichard, 1993). In addition, the number of specific binding sites for [^{3}H]-des-Arg^9-BK and [^{3}H]-des-Arg^{10}-KD in vascular tissues from the rabbit was reported to increase with increasing time of incubation (Barabé *et al.*, 1982) or following treatment with inflammatory mediators such as cytokines (Schneck *et al.*, 1993; Galizzi *et al.*, 1994). Therefore, *de novo* synthesis of B_1 receptors appears to be involved in the induction of vascular responsiveness to des-Arg^9-BK following vascular trauma.

Isolated coronary arteries from a number of species relax in an endothelium-dependent manner to BK (Beny *et al.*, 1987; Toda *et al.*, 1987; Baydoun & Woodward, 1991; Mombouli *et al.*, 1992; Auch-Schwelk *et al.*, 1993; Kilpatrick & Cocks, 1994; Holzmann *et al.*, 1994; Stork & Cocks, 1994), yet do not relax in response to des-Arg^9-BK (Beny *et al.*, 1987; Baydoun & Woodward, 1991; Auch-Schwelk *et al.*, 1993; Holzmann *et al.*, 1994). These findings suggest the presence of B_2 but not B_1 receptors on the endothelium of the coronary vasculature. In the present study we developed a sensitive and robust bioassay for kinin-mediated relaxations in the bovine left anterior descending coronary artery (LAD). Our results show that this tissue contains constitutive B_2 as well as inducible B_1 receptors, both of which mediate endothelium-dependent relaxation partly via the release of nitric oxide (NO; for review see Moncada *et al.*, 1990). Also, the bovine LAD appears to contain a second population of B_1 receptors which mediate direct smooth muscle contractions.

Methods

Preparation of the assay tissue

Sections of bovine myocardium containing the LAD were obtained from a local abattoir (J.R. Ralph & Sons, Pt. Melb., VIC) and immediately placed in chilled (4°C) Krebs solution (composition in mM; Na^+ 143.1, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127.8, HCO_3^- 25.0, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2, glucose 11.0 and ethylenediaminetetraacetic acid 0.1). The LAD was then isolated, cleared of connective tissue and fat and cut into ring segments 3–5 mm in length. Some rings of artery had their endothelium removed by gently abrading the luminal surface with a Krebs-moistened, tapered, wooden stick. Each artery ring was then suspended between two stainless steel wire hooks, one of which was connected to a force displacement transducer (model FT03C, Grass, Quincy, MA, U.S.A.) and the other to a micrometer-adjustable support leg. Preparations were subsequently submerged in water-jacketed 30 ml organ baths containing carbogenated (95% O_2, 5% CO_2) Krebs solution (pH 7.4) maintained at 37°C. All tissues were treated with the cyclo-oxygenase inhibitor indomethacin (3 μM). Some tissues were continuously exposed to either the protein translation inhibitor, cycloheximide (30 and 100 μM) or the transcription inhibitor, actinomycin D (2 μM) from the moment they were placed in the organ bath and then throughout the remainder of the experiment. Changes in isometric, circumferential force were amplified and displayed on dual-channel, flat-bed recorders (W & W Scientific Instruments, Basel, Switzerland).

After a 25 min equilibration period, rings were stretched to 5 g passive force and allowed to recover for 25 min, after which time they were again stretched to 5 g. After a further 25 min, rings of artery were contracted with an isotonic, high potassium physiological salt solution (KPSS) in which all of the NaCl of normal Krebs was replaced with KCl (composition of KPSS in mM; K^+ 124.9, Na^+ 25.0, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25.0, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2, glucose 6.1 and ethylenediaminetetraacetic acid 0.1). When the tissues contracted to a steady plateau the bathing solution was changed to normal Krebs solution and the tissues allowed to return to resting levels of passive force.

Responses to BK, des-Arg^9-BK and ACh after 3 h and 6 h of in vitro *incubation*

After the maximum contraction to KPSS ($KPSS_{max}$), tissues were contracted to approximately 40% of their respective $KPSS_{max}$ with the thromboxane A_2-mimetic, U46619 (1–30 nM). Preliminary studies in our laboratory showed that levels of precontraction above 40% $KPSS_{max}$ reduced the sensitivity and maximum relaxations to both endothelium-dependent and -independent relaxing agents (Kilpatrick, Cocks & Drummond, unpublished data). Once a stable level of active force was reached and after 3 h of total incubation time, responses to cumulative, 0.5 log molar additions of either BK, des-Arg^9-BK or ACh were obtained. After a supramaximal response to each agonist was reached, tissues were washed with fresh Krebs solution and allowed to return to baseline levels of resting force.

After 4.5 h of incubation the same tissues were maximally contracted for a second time with KPSS, washed with normal Krebs solution and allowed to return to baseline levels of resting force. Tissues were again contracted with U46619 to approximately 40% of the second $KPSS_{max}$ and after 6 h of incubation, responses to cumulative, 0.5 log molar additions of either BK, des-Arg^9-BK or ACh were again obtained.

Effects of L-NOARG, L-arginine, des-Arg^9-[Leu^8]-BK *and Hoe-140*

In a separate series of experiments, tissues were contracted twice with KPSS as above but were not exposed to either BK, des-Arg^9-BK or ACh at 3 h of incubation. Following contraction with U46619 to approximately 40% of the second $KPSS_{max}$, some tissues were treated with either the competitive B_1 receptor antagonist, des-Arg^9-[Leu^8]-BK (1, 3, 10 and 30 μM) or the competitive B_2 receptor antagonist, Hoe-140 (1, 3, 10, 30 and 100 nM) and left for at least 20 min. Other tissues were treated for at least 40 min with either the NO synthase inhibitor, N^G-nitro-L-arginine (L-NOARG, 100 μM; Moore *et al.*, 1990), or a combination of L-NOARG (100 μM) and L-arginine (10 mM) prior to contraction to approximately 40% of the second $KPSS_{max}$ with U46619. In all cases, after 6 h of total *in vitro* incubation time, tissues were treated with cumulative 0.5 log molar additions of either BK or des-Arg^9-BK.

Statistics

All cumulative concentration-relaxation curves were normalized as percentages of relaxation from the initial U46619-induced precontraction level. Concentration-dependent contractions (e.g. des-Arg^9-BK in endothelium-denuded rings) as well as U46619-induced levels of precontraction force were expressed as percentages of the respective $KPSS_{max}$ for that tissue. Each normalized curve was then computer-fitted (Graphpad Prism, version 1.00) with a sigmoidal regression curve of the following equation,

$$Y = BOTTOM + (TOP - BOTTOM)/1 + 10^{(pD_2 - X).Hillslope}$$

where X is the logarithm of the agonist concentration and Y is the response. BOTTOM is the lower response plateau. TOP is the upper response plateau and pD_2 is the X value when the response is halfway between BOTTOM and TOP. The variable Hillslope controls the slope of the curve. Mean pEC_{50} values and maximum responses (R_{max}) and their standard errors were then calculated for each response curve. Values of pA_2 were obtained by Schild plot analysis (Arunlakshana & Schild, 1959). Values of *n* refer to numbers of rings of artery each from separate animals. Differences in mean pEC_{50} and R_{max} values within a single tissue (e.g. responses at 3 h versus 6 h) were tested for significance by means of two-tailed paired *t* tests. Differences in mean pEC_{50} and R_{max} values between two experimental groups (e.g. des-Arg^9-BK in the presence and absence of cycloheximide) were tested for significance by means

of two-tailed unpaired t tests. Differences in mean pEC_{50} and R_{max} values as well as mean levels of U46619-induced active force within each experimental group were analysed by one way analysis of variance (ANOVA) with multiple comparisons via Tukey Kramer's modified t statistic. All differences were accepted as significant at the $P<0.05$ level.

Drugs and their sources

The following drugs were used: U46619 (1,5,5-hydroxy-11,9-(epoxymethano)prosta-5Z,13E-dienoic acid; Upjohn, Kalamazoo, U.S.A.), acetylcholine chloride, bradykinin triacetate, cycloheximide, des-Arg^9-bradykinin triacetate, des-Arg^9-[Leu^8]-bradykinin triacetate, indomethacin, L-arginine, N^G-nitro-L-arginine (all from Sigma, MO, U.S.A.), Hoe-140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin; Hoechst, Vic., Aust.) and actinomycin D (Boehringer Mannheim Biochemica, Germany).

Stock solutions of actinomycin D (1 mM) and U46619 (1 mM) were made up in absolute ethanol while those of indomethacin (100 mM) and L-NOARG (100 mM) were made up in Na_2CO_3 (1 M) and $NaHCO_3$ (1 M), respectively. All subsequent dilutions of these drug stocks were in distilled water. All other drugs were made up in distilled water.

Results

Kinin receptors mediating relaxation responses

The B_1-receptor agonist, des-Arg^9-BK (1–100 nM), caused concentration-dependent relaxations (pEC_{50}, 8.00±0.08; R_{max}, 93.9±1.9%) in rings of endothelium-intact bovine LAD that had been incubated for 6 h and contracted to 42.6±0.9% $KPSS_{max}$ with U46619 (Figure 1). The sensitivity (pEC_{50}) but not R_{max} to des-Arg^9-BK was decreased in a concentration-dependent manner by the selective B_1-receptor antagonist, des-Arg^9-[Leu^8]-BK (Figure 2a). Schild analysis gave a pA_2 value for des-Arg^9-[Leu^8]-BK of 6.27±0.11 with a slope of 0.84±0.06 which was not significantly different from unity (Figure 2b). The relaxation curve to des-Arg^9-BK was unaffected by the B_2-receptor antagonist, Hoe-140 (0.1 μM; Figure 2a).

The B_2-receptor agonist, BK, also caused concentration-dependent relaxations (pEC_{50}, 9.75±0.07; R_{max}, 100.1±0.7%) of U46619-contracted (40.5±1.6% $KPSS_{max}$), endothelium-intact rings of bovine LAD incubated for 6 h (Figure 1). Neither the pEC_{50} nor R_{max} to BK was affected by des-Arg^9-[Leu^8]-BK (10 μM), whereas the pEC_{50} but not R_{max} was decreased in a concentration-dependent manner by Hoe-140 (Figure 3a). Schild analysis gave a pA_2 value for Hoe-140 of 9.63±0.14 with a regression slope of 0.93±0.11 which was not significantly different from one (Figure 3b).

Endothelium- and NO-dependence of relaxations to kinins

L-NOARG (100 μM) markedly inhibited relaxation responses to des-Arg^9-BK (Figure 4a). In the presence of L-NOARG the response to des-Arg^9-BK was biphasic, lower concentrations of des-Arg^9-BK (1–10 nM) caused concentration-dependent contractions whilst higher concentrations of des-Arg^9-BK (30–1000 nM) caused concentration-dependent relaxations. The maximum relaxation response to des-Arg^9-BK was significantly reduced by about 40% in L-NOARG-treated tissues compared to controls. The inhibitory effect of L-NOARG (100 μM) on des-Arg^9-BK-induced relaxations was not seen in tissues treated with a 100 fold excess of L-arginine (Figure 4a). L-NOARG (100 μM) also caused a significant 2 fold decrease in the pEC_{50} for BK although it had no effect on R_{max} (Figure 4b). L-Arginine (10 mM) reversed this inhibitory effect of L-NOARG.

Mechanical removal of the endothelium abolished relaxations to both des-Arg^9-BK (Figure 4a) and BK (Figure 4b) and revealed concentration-dependent, tonic contractions to des-Arg^9-BK only (pEC_{50}, 7.57±0.09; R_{max}, 83.4±9.1%).

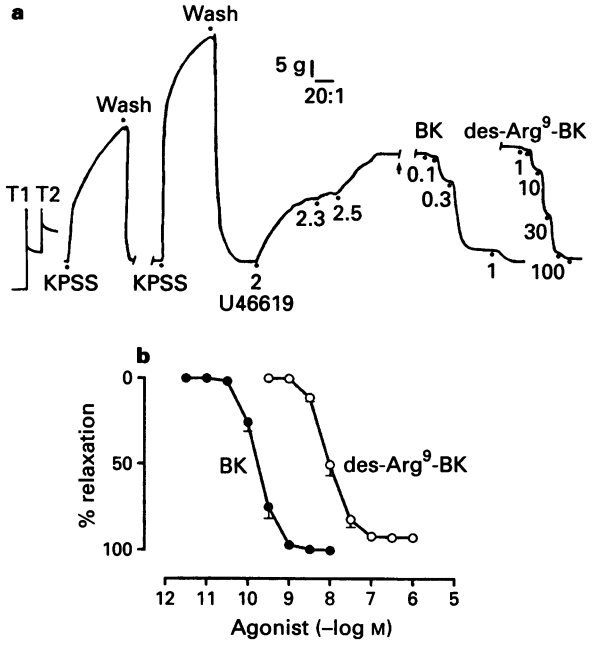

Figure 1 (a) Traces of typical recordings showing both the procedure used for optimizing relaxation conditions as well as relaxation responses to BK and des-Arg^9-BK in ring segments of endothelium-intact bovine LAD. After two stretches to 5 g (T1 and T2) and then two maximum contractions with 125 mM K^+ Krebs solution (KPSS), rings were contracted with U46619 (1–30 nM) to approximately 40% of their respective maximum contraction to the second KPSS treatment ($KPSS_{max}$). At 6 h after set up, rings were treated with cumulatively increasing 0.5 log M concentrations of either BK or des-Arg^9-BK. Note that the gain was reduced after T2 and for clarity the U46619 contraction for des-Arg^9-BK was omitted. Time calibration bar indicates 20 min and 1 min, before and after the arrow, respectively. Concentrations are expressed as nanomolar. (b) Group data from experiments depicted in (a). Values [mean± s.e.mean from 11 (BK) and 17 (des-Arg^9-BK) experiments] are expressed as percentages of relaxation from the U46619-induced force (BK, 40.5±1.6% $KPSS_{max}$; des-Arg^9-BK, 42.6±0.9% $KPSS_{max}$).

Time-dependent sensitization of responses to des-Arg^9-BK

Relaxations to des-Arg^9-BK increased in sensitivity and maximum from 3 h (pEC_{50}, 7.45±0.10; R_{max}, 84.6±3.3%) to 6 h (pEC_{50}, 7.94±0.06, $P<0.0001$; R_{max}, 91.4±2.5%; $P<0.05$) (Figure 5a). There were no significant differences between the U46619-induced precontraction levels at 3 h (43.4±2.4% $KPSS_{max}$) and 6 h (41.2±1.5% $KPSS_{max}$). Also, responses to des-Arg^9-BK after 6 h in these vessels were not significantly different from the 6 h-response in vessels that were not previously relaxed at 3 h with des-Arg^9-BK (compare Figures 1b and 5a).

Unlike the response to des-Arg^9-BK, relaxations to BK showed no changes in either sensitivity or maximum from 3 h (pEC_{50}, 9.82±0.09; R_{max} 99.7±0.6%) to 6 h (pEC_{50}, 9.84±0.06; R_{max}, 100.5±0.8%) (Figure 5b). Similarly, responses to another endothelium-dependent relaxing agent, acetylcholine, were identical in terms of sensitivity and maximum at 3 h (pEC_{50}, 8.29±0.03; R_{max} 99.0±0.4%) and 6 h (pEC_{50}, 8.30±0.09, R_{max}, 98.5±1.2%) (Figure 5c).

Finally, experiments were performed in endothelium-denuded rings to determine whether contractions to des-Arg^9-BK displayed a similar sensitization process to that observed with endothelium-dependent relaxations to des-Arg^9-BK (Figure 6). After 3 h of *in vitro* incubation, des-Arg^9-BK caused concentration-dependent contractions of endothelium-denuded

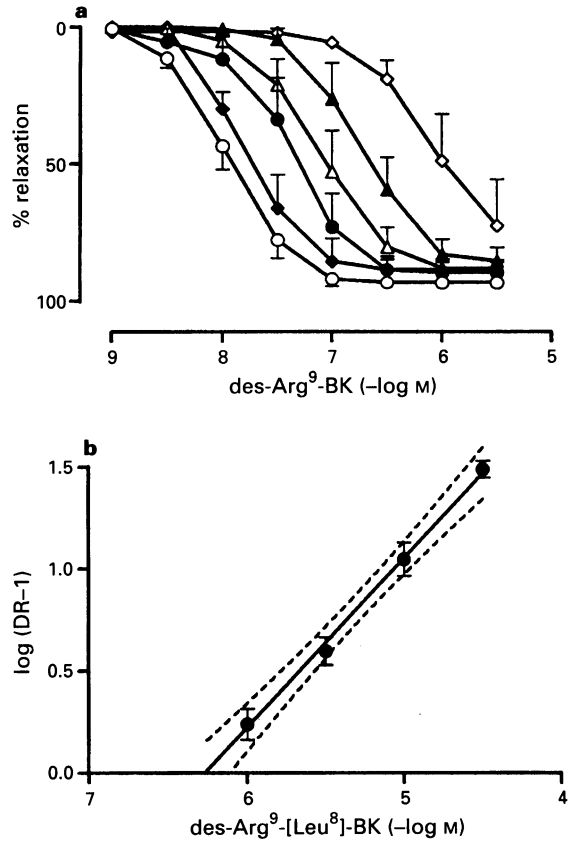

Figure 2 (a) Cumulative concentration-relaxation curves to des-Arg^9-BK alone (○; $n=11$) and in the presence of 1 (●; $n=5$), 3 (Δ; $n=5$), 10 (▲; $n=5$) and 30 μM (◇; $n=4$) des-Arg^9-[Leu^8]-BK, and Hoe-140 (100 nM; ◆; $n=6$) in rings of endothelium-intact bovine LAD incubated for 6 h at 37°C. Corresponding levels of initial active force to U46619 (expressed as percentages of maximum contraction to KPSS) were (○) 43.6 ± 1.3%, (●) 40.5 ± 2.5%, (△) 39.2 ± 1.3%, (▲) 41.1 ± 3.1%, (◇) 36.6 ± 2.7% and (◆) 40.9 ± 3.6%. These values were not significantly different from one another. Values (mean ± s.e.mean) are expressed as percentages of relaxation from the U46619-induced force. (b) Schild plot demonstrating competitive antagonism of the response to des-Arg^9-BK by des-Arg^9-[Leu^8]-BK. Values represent mean ± s.e.mean. $pA_2 = 6.27 \pm 0.11$; $r = 0.84 \pm 0.06$. Dotted lines indicate 95% confidence limits.

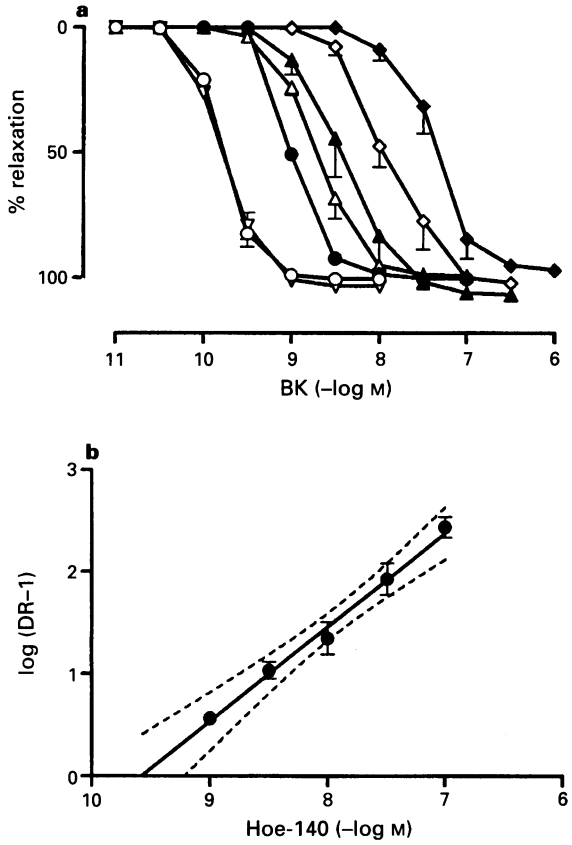

Figure 3 (a) Cumulative concentration-relaxation curves to BK alone (○; $n=6$) and in the presence of 1 (●; $n=1$), 3 (△; $n=4$), 10 (▲; $n=4$), 30 (◇; $n=4$) and 100 nM (◆; $n=3$) Hoe-140, and des-Arg^9-[Leu^8]-BK (10 μM; ▽; $n=4$) in rings of endothelium-intact bovine LAD incubated for 6 h at 37°C. Corresponding levels of initial active force to U46619 (expressed as percentages of the maximum contraction to KPSS) are (○) 37.6 ± 2.5, (●) 37.9 ± 0.0%, (△) 37.28 ± 2.0%, (▲) 34.4 ± 1.2%, (◇) 33.4 ± 1.9%, (◆) 35.4 ± 5.4% and (▽) 36.9 ± 2.4%. These values were not significantly different from one another. Values are expressed as percentages of relaxation from the U46619-induced force. (b) Schild plot demonstrating competitive antagonism of the response to BK by Hoe-140. Values represent mean ± s.e.mean. $pA_2 = 9.63 \pm 0.14$; $r = 0.93 \pm 0.11$. Dotted lines indicate 95% confidence limits.

vessels (pEC_{50}, 7.37 ± 0.08; R_{max}, 126.8 ± 8.5%). After 6 h and in the same tissues, a small but significant ($P<0.05$) increase in sensitivity to des-Arg^9-BK was detected (pEC_{50}, 7.62 ± 0.12). By contrast, the maximum response to des-Arg^9-BK after 6 h (R_{max}, 103.3 ± 8.6%) was significantly ($P<0.05$) less than that observed at 3 h.

Effect of protein synthesis inhibition on responses to des-Arg^9-BK and BK

Des-Arg^9-BK caused a biphasic response in tissues continuously exposed to cycloheximide (30 μM) and incubated for 3 h (Figure 7a). Lower concentrations of des-Arg^9-BK (3–30 nM) contracted the tissues, whilst higher concentrations (0.1–3 μM) caused relaxations. The maximum relaxation to des-Arg^9-BK after 3 h, however, was significantly less ($P<0.05$) in tissues treated with cycloheximide (R_{max}, 40.4 ± 15.4%) than it was in control tissues (R_{max}, 86.5 ± 3.0%). Increasing the concentration of cycloheximide to 100 μM appeared both to increase the magnitude of contractions to lower concentration of des-Arg^9-BK, as well as further reducing the maximum relaxation; however, these differences were not significant.

After 6 h of incubation with 30 μM cycloheximide the pEC_{50} (7.62 ± 0.15) and R_{max} (100.81 ± 2.57%) values for des-Arg^9-BK were not significantly different from those obtained in 6 h control tissues (pEC_{50}, 7.91 ± 0.06; R_{max} 92.5 ± 2.8%; Figure 7b). By contrast, in tissues treated with 100 μM cycloheximide, the pEC_{50} for des-Arg^9-BK after 6 h was significantly reduced (pEC_{50}, 6.57 ± 0.32; $P<0.001$) compared to 6 h control values (pEC_{50}, 7.91 ± 0.06). There was, however, no significant difference in maximum relaxation after 6 h between control (R_{max}, 92.5 ± 2.8%) and 100 μM cycloheximide-treated (R_{max}, 83.8 ± 7.8%) tissues.

A similar biphasic response to des-Arg^9-BK was observed in tissues treated with 2 μM actinomycin D (Figure 7c). Although a pEC_{50} value could not be obtained for the relaxation component due to poor curve fitting, the sensitivity to des-Arg^9-BK at 3 h in actinomycin D-treated tissues appeared to be reduced by approximately 30 fold compared to the 3 h control des-Arg^9-BK response. The maximum relaxation to des-Arg^9-BK after 3 h in actinomycin D-treated tissues (R_{max}, 64.0 ± 15.8%), however, was not significantly different from that seen in control tissues (R_{max}, 85.44 ± 7.64%). After 6 h of incubation with actinomycin D, analysis of the relaxation component of the biphasic response to des-Arg^9-BK produced a pEC_{50} value of 6.04 ± 0.04, which was significantly ($P<0.0001$) and approximately 100 fold less than the pEC_{50} (7.95 ± 0.11) obtained in 6 h control tissues (Figure 7d). Again, there was no significant difference between the 6 h R_{max} to des-Arg^9-BK in actinomycin D-treated (90.8 ± 2.1%) and control (87.9 ± 6.1%) tissues.

Unlike the response to des-Arg^9-BK, BK-induced relaxations after 3 h were unaffected by cycloheximide (100 μM; Figure 7e).

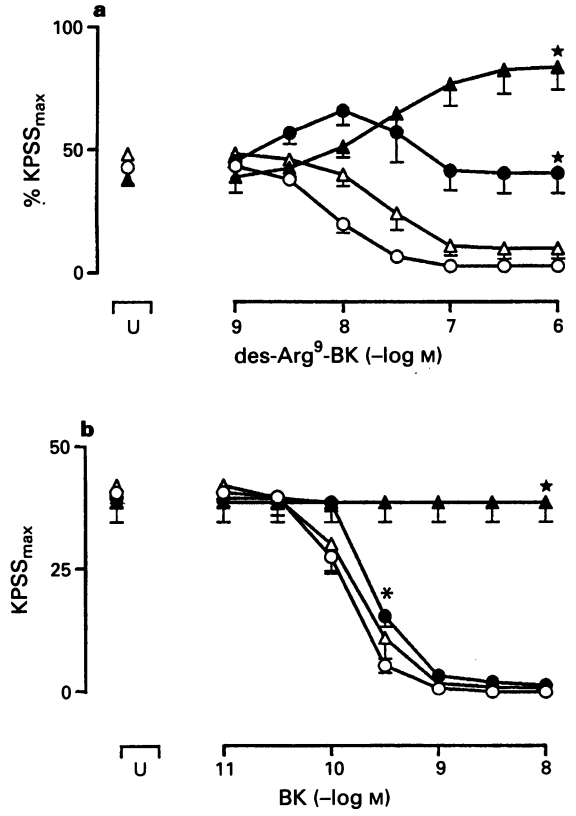

Figure 4 Effect of L-NOARG (0.1 mM; ●), L-NOARG (0.1 mM) in the presence of L-arginine (10 mM; △) and after endothelium removal (▲) on responses to (a) des-Arg^9-BK and (b) BK in rings of bovine LAD. Control responses are represented by (○). Rings of artery were incubated for 6 h at 37°C and precontracted with U46619 (U) to approximately 40% of their respective maximum contraction to KPSS ($KPSS_{max}$). In (a) maximum responses (R_{max}) were compared statistically while in (b) comparisons were made between both pEC_{50} and R_{max} values for each group. The symbols (*) and (*) indicate significant differences between pEC_{50} and R_{max} values, respectively, in control and experimental groups (two-tailed P value; for Tukey-Kramer's test for multiple comparisons after one-way ANOVA). Values (mean ± s.e.mean from 4 to 10 experiments) are expressed as a percentages of $KPSS_{max}$.

Discussion

In this study we have characterized both B_2 and B_1 kinin receptors on endothelial cells of the bovine LAD mediating endothelium-dependent relaxation partially via the release of NO. Thus, relaxations to des-Arg^9-BK and BK were (1) abolished by endothelium removal, (2) competitively and selectively antagonized by the B_1 and B_2 receptor antagonists des-Arg^9-[Leu^8]-BK and Hoe-140, respectively, with apparent affinity (pA_2) values similar to those reported for other tissues containing B_1 (Regoli *et al.*, 1977; Churchill & Ward, 1986) and B_2 (Hock *et al.*, 1991) receptors and (3) partially blocked by L-NOARG. In rings of artery denuded of endothelium, des-Arg^9-BK but not BK caused concentration-dependent contractions which, based on order of agonist potencies for kinins (Regoli & Barabé, 1980), suggests the presence of B_1 receptors on the smooth muscle layer of the bovine LAD.

The ability of BK to cause endothelium-dependent relaxations of isolated coronary arteries from man (Auch-Schwelk *et al.*, 1993; Stork & Cocks, 1994) and other species (Beny *et al.*, 1987; Toda *et al.*, 1987; Mombouli *et al.*, 1992; Auch-Schwelk *et al.*, 1993; Kilpatrick & Cocks, 1994; Holzmann *et al.*, 1994) is well established. By contrast, although Staszewska-Woolley & Woodman (1991) reported an increase in coronary blood flow in the anaesthetized dog following intracoronary des-Arg^9-BK infusion, studies on isolated coronary arteries have demonstrated either contractions or no response to des-Arg^9-BK. Thus, isolated bovine (Auch-Schwelk *et al.*, 1993; Holzmann *et al.*, 1994) and porcine (Beny *et al.*, 1987) coronary arteries relaxed in an endothelium-dependent manner to BK, yet only contracted to des-Arg^9-BK. While the discrepancy between our results and those in porcine vessels may be due to species differences in kinin receptor profiles in the coronary vasculature, the opposing results of Auch-Schwelk *et al.* (1993) and Holzmann *et al.* (1994) in bovine coronary arteries are more difficult to explain. One explanation may be differences in the degree of precontraction used in each study. Unlike our study where contractions were normalized to optimally measure relaxation responses, Auch-Schwelk *et al.* (1993) and Holzmann *et al.* (1994) used single, high concentrations of either prostaglandin $F_{2\alpha}$ or U46619 with no reference as to what these precontraction levels were when expressed as a percentage of the maximum contraction for each individual tissue. Thus, the sensitivity of BK reported by Auch-Schwelk *et al.* (1993) and Holzmann *et al.* (1994) was less than that obtained in our study which may indicate differences in the levels of functional antagonism between the studies. In the present study, des-Arg^9-BK was approximately two orders of magnitude less potent than BK as an endothelium-dependent relax-

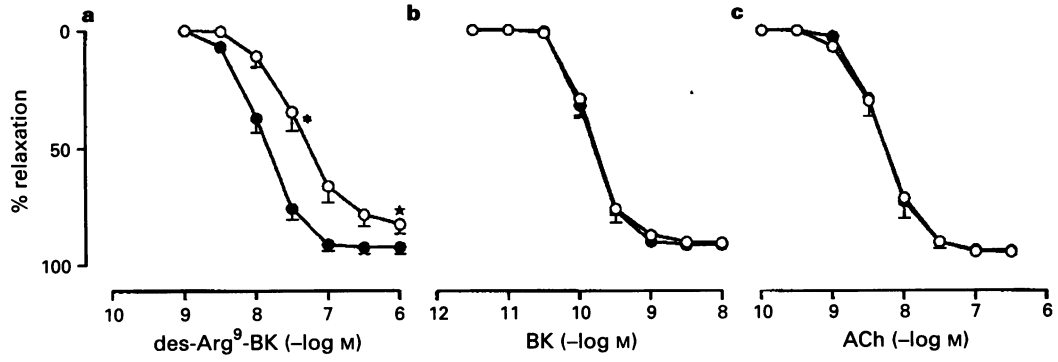

Figure 5 Response to (a) des-Arg^9-BK ($n=17$), (b) BK ($n=6$) and (c) ACh ($n=4$) after 3 h (○) and 6 h (●) of *in vitro* incubation in endothelium-intact rings of bovine LAD. Precontraction levels to U46619 for each group (expressed as percentages of the maximum contraction to KPSS) at 3 h and 6 h respectively were for (a) (○) 43.4 ± 2.4% and (●) 41.2 ± 1.5%, for (b) (○) 42.9 ± 3.1% and (●) 41.3 ± 2.6% and for (c) (○) 42.7 ± 2.1% and (●) 41.1 ± 1.7%. Note that there were no significant differences between levels of initial active force at 3 h and 6 h for any of these groups. Symbols (*) and (*) represent significant differences between pEC_{50} and R_{max} values respectively at 3 h and 6 h of incubation (two-tailed P value <0.05; Student's paired t test). Values (mean ± s.e.mean) are expressed as percentages of relaxation from the U46619-induced tone.

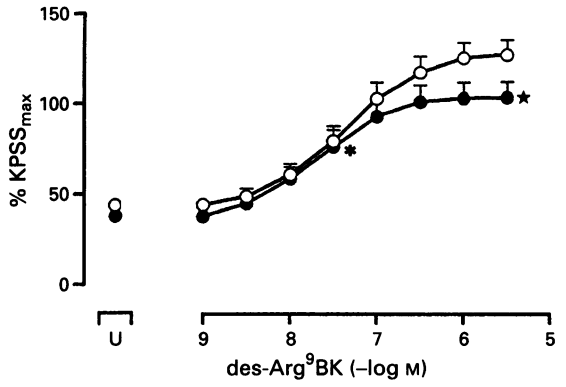

Figure 6 The effect of des-Arg^9-BK after 3 h (○) and 6 h (●) of incubation in endothelium-denuded rings of bovine LAD precontracted to approximately 40% of their respective maximum contraction to KPSS ($KPSS_{max}$) with U46619 (U). Note, the initial levels of precontraction (expressed as percentages of $KPSS_{max}$) for the 3 h and 6 h group were 43.8 ± 3.3% and 37.9 ± 2.1%, respectively, and these values were not significantly different from one another. Symbols (*) and (★) represent significant differences between pEC_{50} and R_{max} values respectively at 3 h and 6 h of incubation (two-tailed P value <0.05; Student's paired t test). Values (mean ± s.e.mean from 6 experiments) are expressed as percentages of $KPSS_{max}$.

ing agent, which in part, may have been due to the opposing smooth muscle contractile effects of des-Arg^9-BK. Therefore, any further increase in the degree of functional antagonism produced by a precontracting agent would be expected to affect relaxations to des-Arg^9-BK to a greater extent than to BK.

Both BK and des-Arg^9-BK stimulate the production of NO in cultured endothelial cells (Wiemer & Wirth, 1992; Drummond & Cocks, unpublished observations). In the present study, L-NOARG reduced the sensitivity of relaxations to BK as well as both the sensitivity and maximum relaxation to des-Arg^9-BK. Inhibition of relaxations to both agonists by L-NOARG was specific in that it was reversed in the presence of an excess of L-arginine. Therefore, NO-release appeared to account, at least in part, for the endothelium-dependent relaxation response to both des-Arg^9-BK and BK in the bovine LAD.

The L-NOARG-resistant component of the kinin-induced relaxations, in particular to BK, may indicate either incomplete block of NO synthase by L-NOARG or alternatively may represent the contribution of other non-NO, endothelium-derived relaxing factors to the overall relaxation response. In support of the former explanation, Martin *et al.* (1992) demonstrated that the degree of inhibition of muscarinic-receptor mediated relaxations in the rabbit jugular vein by the methyl ester of L-NOARG, L-NAME, was greater for agonists with a low 'reserve' (i.e. partial agonists) than it was for those with a higher reserve (i.e. full agonists). Therefore, Martin *et al.* (1992) concluded that tissue- and agonist-dependent differences in the ability of L-arginine analogues to inhibit endothelium-dependent relaxations are related to differences in the efficiency of occupancy-effect coupling ('reserve') for each receptor system studied. Thus, in the present study the higher degree of L-NOARG inhibition seen with des-Arg^9-BK as compared to BK may indicate a low reserve for the former agonist.

An alternative to the reserve theory of Martin *et al.* (1992) is that if NO production is abolished by L-NOARG and L-NAME, then other mechanism/s or factor/s may be responsible for L-arginine analogue-resistant relaxations to endothelium-dependent agonists (see Cowan *et al.*, 1993; Kilpatrick & Cocks, 1994). Although cyclo-oxygenase products such as prostacyclin are released from endothelial cells in response to both B_1 and B_2 receptor stimulation (D'Orleans-Juste *et al.*, 1989), it is unlikely that prostanoids contributed to the L-arginine analogue-resistant relaxations to des-Arg^9-BK and BK seen here in the bovine LAD since all experiments were carried out in the presence of indomethacin. Other candidates for non-NO relaxing factors include endothelium-derived hyperpolarizing factor (EDHF; for reviews see Nagao & Vanhoutte, 1993; Garland *et al.*, 1995) and cytochrome P450 metabolites of arachidonic acid (Pinto *et al.*, 1987; Rosolowsky & Campbell, 1993).

In this study we demonstrated an increase in both the sensitivity and maximum relaxation to des-Arg^9-BK in endothelium-intact arteries from 3 h to 6 h. The increased responsiveness at 6 h of *in vitro* incubation was not due to prior exposure to des-Arg^9-BK at 3 h since the response at 6 h was identical in tissues exposed or not exposed previously to des-Arg^9-BK. It is also unlikely that the increase in sensitivity to des-Arg^9-BK was due to a decrease in its degradation by angiotensin converting enzyme (ACE) at 6 h because ACE has an equivalent or higher specificity for BK (see Ward, 1991) yet the response to BK remained unchanged with time. One explanation for the increase in sensitivity to des-Arg^9-BK at 6 h is that B_1 receptors were synthesized *de novo* during the incubation period as initially proposed by Regoli *et al.* (1978). In the present study, both the protein translation inhibitor, cycloheximide, and the transcription inhibitor, actinomycin D, selectively attenuated responses to des-Arg^9-BK at 3 h and 6 h. Whilst this result supports the hypothesis that B_1 receptor proteins were synthesized during the incubation period, we cannot rule out the possibility that cycloheximide and actinomycin D acted on transduction mechanisms 'downstream' of the receptor. Thus, the apparent increase in B_1 receptor affinity with time may have been due to changes in occupancy-effector coupling mechanisms rather than the *de novo* synthesis of new receptors.

Irrespective of their precise mechanism of action, neither cycloheximide nor actinomycin D appeared to be totally effective in inhibiting the induction of responsiveness to des-Arg^9-BK. The block by cycloheximide appeared to be incomplete for two reasons, (1) the increase in sensitivity of des-Arg^9-BK persisted in tissues treated with cycloheximide and (2) a higher concentration of cycloheximide improved the degree of block as shown by the further reduction in des-Arg^9-BK sensitivity at 6 h. Unlike cycloheximide, the inhibitory actions of actinomycin D appeared to be irreversible since responses to des-Arg^9-BK did not improve between 3 h and 6 h of incubation. The near maximum relaxations to high concentrations of des-Arg^9-BK in actinomycin D-treated tissues at both 3 h and 6 h were unlikely to be due to B_2 receptor activation nor could they solely be explained by a constitutive component of the B_1 response since they were not observed in tissues treated with cycloheximide at 3 h. These relaxations may, however, be explained if actinomycin D had a slow onset of action, such that the response remaining to des-Arg^9-BK was mediated by B_1 receptors upregulated before actinomycin D was able to block the upregulation process.

The incomplete block of the response to des-Arg^9-BK by both cycloheximide and actinomycin D appears to contradict the findings of Regoli *et al.* (1978), Bouthillier *et al.* (1987), Deblois & Marceau (1987) and Deblois *et al.* (1989). These authors each demonstrated virtual abolition of the response to a single concentration of des-Arg^9-BK (ranging from 0.1–1.3 μM) following continuous exposure to either cycloheximide or actinomycin D. In the present study the effects of protein synthesis inhibition on the response to des-Arg^9-BK over the entire concentration-range were examined. If, as in previous studies (Regoli *et al.*, 1978; Bouthillier *et al.*, 1987; Deblois & Marceau, 1987; Deblois *et al.*, 1989), we had tested the effects of either cycloheximide or actinomycin D against a single concentration of des-Arg^9-BK (for example 0.1 μM which we showed to induce near maximum relaxations at 3 h and 6 h in control tissues) we too may have concluded that these compounds abolished the upregulation of responsiveness to des-Arg^9-BK. In examining the effects of protein synthesis inhibitors over the whole concentration-range of des-Arg^9-BK, our study has provided an insight into the high degree of 're-

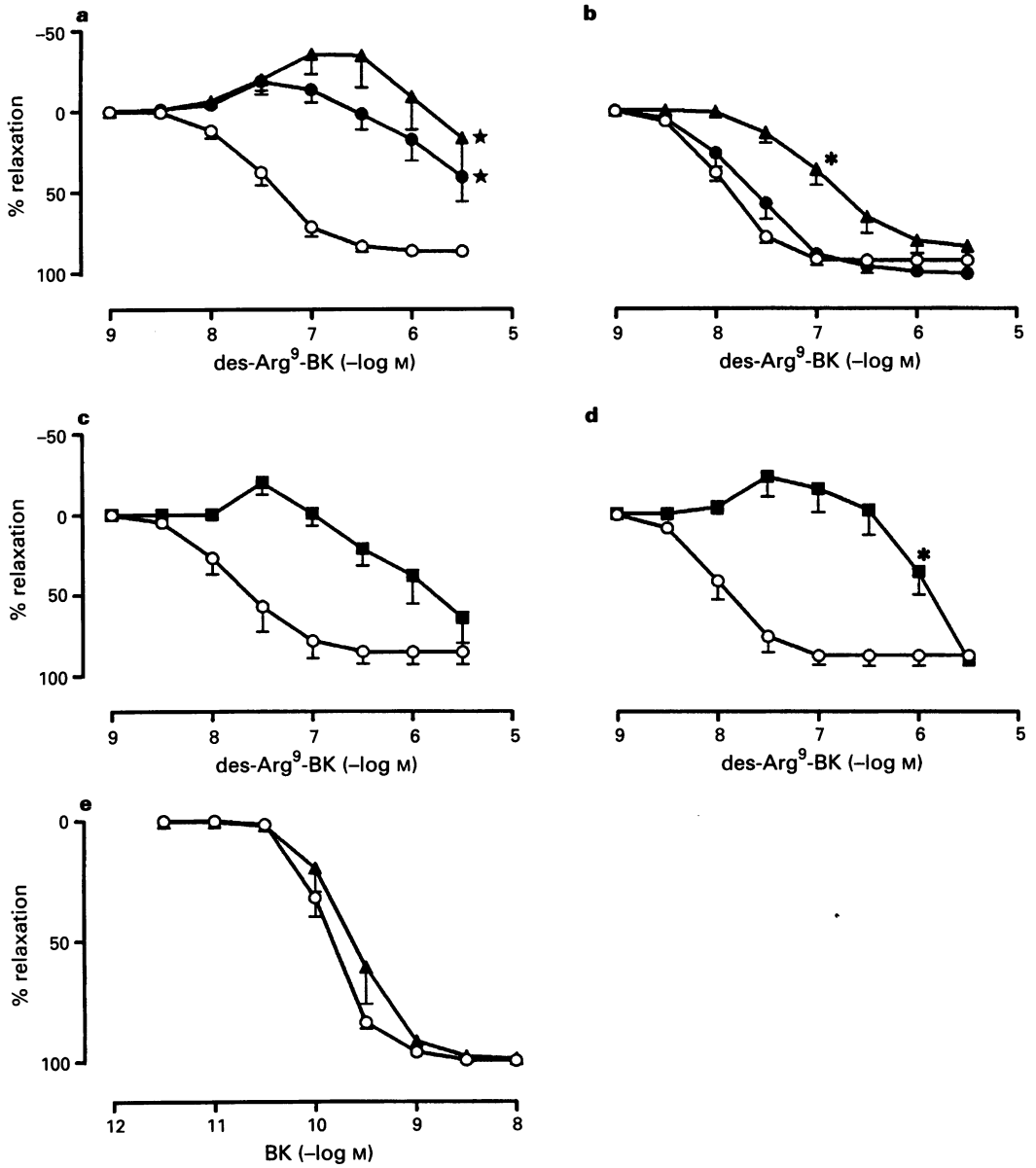

Figure 7 The effect of (a, b, e) cycloheximide (○, control; ●, 30 μM; ▲, 100 μM; $n=5$ to 7) and (c, d) actinomycin D (○, control; ■, 2 μM; $n=5$) on relaxation responses to des-Arg^9-BK and BK in ring segments of bovine LAD after (a, c, e) 3 h and (b, d) 6 h of *in vitro* incubation. Symbols (*) and (*) show differences between control and experimental pEC_{50} and R_{max} values, respectively ($P<0.05$; Student's unpaired t test or Tukey-Kramer's test for multiple comparisons after one-way ANOVA). Note pEC_{50} values for des-Arg^9-BK in the presence of both cycloheximide (30 and 100 μM) after 3 h, and actinomycin D after 3 h could not be obtained. Also, there were no significant differences between the levels of precontraction to U46619 (1–30 nM) in any of these groups (data not shown). Values (mean ± s.e.mean) are expressed as percentages of relaxation from the U46619-induced tone.

serve' associated with the des-Arg^9-BK/B_1 interaction in this tissue, in that, despite the reduction in the number or affinity of functional B_1 receptors after 6 h associated with cycloheximide and actinomycin D treatment, des-Arg^9-BK was still able to elicit maximum relaxations at higher concentrations.

The precontraction method utilized to optimize conditions for relaxation in this study allowed us to make direct comparisons of the sensitivity and magnitude of responses to des-Arg^9-BK between 3 h and 6 h of incubation without the interference of functional antagonism. This method did not, however, allow us to construct relaxation curves to des-Arg^9-BK prior to the 3 h time point. This fact, combined with the inability of either cycloheximide or actinomycin D to abolish totally responses to des-Arg^9-BK (as described above) makes it difficult to draw any firm conclusions as to whether relaxation responses to des-Arg^9-BK were entirely inducible or whether they were partially present from the beginning of the experimental period.

Finally, in addition to mediating endothelium-dependent relaxations, des-Arg^9-BK also caused concentration-dependent contractions in endothelium-denuded rings of bovine LAD. The sensitivity of these contractions appeared to increase slightly from 3 h to 6 h of incubation which might indicate an increase in the number or efficiency of transduction coupling mechanisms of smooth muscle B_1 receptors, similar to that observed on endothelial cells. Arguing against this suggestion, however, was the observation that the maximum contraction to des-Arg^9-BK decreased from 3 h to 6 h. Furthermore, contractions to 30 nM des-Arg^9-BK at both 3 h and 6 h in endothelium-intact rings treated with actinomycin D were similar to those observed at 3 h and 6 h in endothelium-denuded rings.

In conclusion, our study has demonstrated that endothelial cells of the bovine LAD contain both B_1 and B_2 kinin receptors, both of which mediate endothelium-dependent re-

laxation. The response to B_2 receptor stimulation appears to be constitutive whereas that to B_1 receptor stimulation is inducible from 3 h to 6 h and may represent the *de novo* synthesis of new receptors during the incubation period. Not only is this the first report of B_1 receptors on coronary endothelial cells but it also demonstrates that the bovine LAD is a robust functional bioassay for endothelial kinin receptors.

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