Femtomolar bradykinin-induced relaxation of isolated bovine coronary arteries, mediated by endothelium-derived nitric oxide

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We reported previously that bradykinin induces endothelium-dependent relaxation at nanomolar (nM) concentrations in isolated bovine coronary arteries with an intact endothelium. Recently we have found that in the presence of 10 μM indomethacin, femtomolar (fM) concentrations of bradykinin induce endothelium-dependent relaxation in some bovine coronary arteries (≈ 10% of the coronary arteries examined). The present study was designed to characterize the relaxation induced by fM bradykinin. Relaxation was completely abolished by repeated application of fM bradykinin, by 100 μM N^{ω}- nitro-L- arginine methyl ester and by 10 μM methylene blue. Relaxation induced by nM bradykinin was partly affected by these treatments. Relaxation induced by both concentrations of bradykinin was inhibited by a B_2-kinin receptor antagonist, [$Thi^{5,8}$, D-Phe^{7}]-bradykinin, in a concentration-dependent manner, but not by a B_1-kinin receptor antagonist, des-Arg^{9}, [Leu^{8}]-bradykinin. In the presence of 10 μM captopril, an angiotensin-converting enzyme (ACE) inhibitor, all coronary arteries examined in this experiment showed endothelium-dependent relaxation to fM bradykinin. These results show that some bovine coronary arteries relax in response to fM bradykinin, and this response is mediated predominantly by the release of nitric oxide via stimulation of endothelial B_2-kinin receptors. The relaxation may be dependent on ACE activity.

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INTRODUCTION

It is well known that bradykinin induces endothelium-dependent relaxation of arteries in a variety of species by releasing endothelium-derived relaxing factors (EDRFs) (Furchgott, 1988). It has been confirmed that the main EDRFs are nitric oxide (NO) (Palmer *et al.*, 1987). Recent studies, however, suggest that NO does not account for all of the endothelium-dependent relaxation evoked by bradykinin (Richard *et al.*, 1990; Myers *et al.*, 1992). In porcine coronary arteries, for example, it has been reported that NO probably does not play a major role in bradykinin-induced endothelium-dependent relaxation (Nagao & Vanhoutte, 1992; Matsumoto *et al.*, 1993). Our previous study also showed that bradykinin evoked endothelium-dependent relaxation in the presence of both indomethacin and L-nitro-arginine in isolated bovine coronary arteries (Obi *et al.*, 1993).

Bradykinin usually induces endothelium-dependent relaxation at nanomolar concentrations in bovine coronary arteries with an intact endothelium (Obi *et al.*, 1993). Recently, we have found that very low (femtomolar) concentrations of bradykinin induce endothelium-dependent relaxation in bovine coronary arteries. The present study was designed to characterize the endothelium-dependent relaxation evoked by fM bradykinin in isolated bovine coronary arteries.

MATERIALS AND METHODS

Coronary arteries from 82 freshly slaughtered oxen (castrated males, about 2.5 years old, Japanese black beef cattle which were killed by the use of a captive bolt pistol) were obtained at an abattoir and transferred to our laboratory immersed in ice-cold Krebs-Ringer bicarbonate solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM glucose), aerated with a mixture of 95% O_2 and 5% CO_2. The left circumflex coronary arteries were carefully dissected out and cleaned of adhering connective tissue. The coronary artery was cut into 3–4 mm rings which were mounted

horizontally in an organ bath filled with 15 mL of Krebs-Ringer bicarbonate solution. The bath solution was maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. The final pH of the solution was 7.4. The coronary ring was attached to a force transducer (TB-611T, Nihon Kohden Kogyo, Co., Tokyo), and isometric force was recorded on a pen recorder (AP-621G, Nihon Kohden Kogyo, Co., Tokyo). Each coronary ring was stretched to an optimal tension of 20 mN, as determined by repeated stimulation with 60 mM KCl. Specimens were allowed to equilibrate for 90–120 min before starting the experiments.

All experiments were performed in the presence of indomethacin (10 μM) to eliminate the effects of prostanoids (Rosolowsky & Campbell, 1993). The ring was precontracted with 3 μM prostaglandin $F_{2\alpha}$(PG $F_{2\alpha}$) before addition of bradykinin. Cumulative concentration-response curves were obtained by making step-wise increases in the concentrations of bradykinin present in the bath; the addition was made as soon as a steady response had been obtained with the previous concentration. When antagonists or metabolic inhibitors were used, each was added to the organ bath 30 min (B_1-or B_2-kinin antagonists, captopril) or 60 min (N^{ω}-nitro-L-arginine methyl ester (L-NAME), methylene blue) before the concentration-response curves of bradykinin were obtained. To remove the endothelium, the intimal surface was gently rubbed with a swab soaked in Krebs-Ringer bicarbonate solution. The absence of endothelium was verified by absence of relaxation after addition of acetylcholine. The log concentration-ratio (CR) of EC_{50} values (i.e. concentration producing 50% maximal response) in the absence or presence of B_2-kinin antagonist was calculated and plotted against the logarithm of antagonist concentration to obtain the pA_2 values (Arunlakshana & Schild, 1959). The pIC_{50} is the negative logarithm of the molar concentration of an antagonist that inhibits 50% maximal response. The pD_2 is the negative logarithm of the molar concentration of bradykinin causing 50% relaxation (EC_{50}).

The following drugs were used: bradykinin, des-Arg^9, [Leu^8]-bradykinin, [Thi^{5,8}, D-Phe^7]-bradykinin, L-NAME, enalapril maleate (Sigma Chemical Company, St. Louis, MO, USA); L-arginine monohydrochloride, D-arginine monohydrochloride (Wako Pure Chemical Industries, Ltd, Inc., Osaka); indomethacin, methylene blue, sodium nitroprusside (Nacalai Tesque, Inc., Kyoto); $PGF_{2\alpha}$ (Ono Pharmaceutical Co., Ltd, Osaka); captopril (Research Biochemicals International, Natick, MA, USA). All drugs were dissolved in distilled water, and kept on ice during an experiment. Concentrations of the drugs are expressed as the final molar concentration in the organ bath.

Results are expressed as mean ± SEM. In rings contracted with $PGF_{2\alpha}$, responses are expressed as a percentage of the relaxation induced by 100 μM nitroprusside (SNP). Unless stated otherwise, *n* refers to the number of animals from which the coronary rings were isolated. Statistical evaluation of the data was performed using Student's paired or unpaired *t*-test, as appropriate. Scheffé's test for multiple comparisons was used to identify differences among values (Wallenstein *et al.*, 1980). Values were considered to be significantly different when *P* was less than 0.05.

RESULTS

Endothelium-dependent relaxation induced by fM bradykinin

Bradykinin evoked a concentration-dependent relaxation of isolated bovine coronary arteries precontracted with 3 μM PG $F_{2\alpha}$. As shown in Fig. 1, bradykinin evoked two types of relaxation: one (A-type: upper tracing) was induced by bradykinin concentrations greater than 100 nM, while the other (B-type: lower tracing) was induced by fM bradykinin. Type B relaxation was observed in ≈ 10% of the coronary arteries studied (45 out of 400 animals). The calculated pD_2 value for bradykinin in the A type response (9.59 ± 0.65, $n = 6$) was significantly ($P < 0.01$) smaller than that for the B type response (12.69 ± 0.28, $n = 6$). Both types of relaxation disappeared in de-endothelialized coronary arteries (Fig. 1. right).

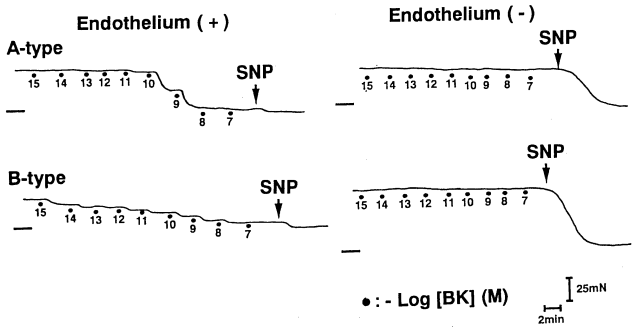

Fig. 1. Typical tracings of bradykinin (BK)-induced relaxation in bovine coronary arteries with (+) and without (−) endothelium partially contracted with 3 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Absolute contrations in A-type coronary arteries ($n = 41$) with and without endothelium were 45.2 ± 1.5 mN and 49.0 ± 1.2mN, respectively, and in B-type arteries ($n = 41$) with and without endothelium were 45.5 ± 1.1 mN and 48.8 ± 1.2mN, respectively. Horizontal lines to the left of each tracing represent the level before addition of $PGF_{2\alpha}$. BK was added to the organ bath at points indicated; numbers indicate the concentration (-logM) of BK. SNP: sodium nitroprusside (100 μM).

Desensitizing effects of repeated application of bradykinin

A preliminary study was undertaken to determine the factors determining coronary artery responsiveness to bradykinin. Cumulative concentration-relaxation curves for bradykinin were constructed at 2-h intervals over an 8-h period. As shown in Table 1, type A responses were not significantly affected by repeated application of bradykinin, while type B responses were significantly attenuated and finally showed type A responses.

Bradykinin receptor subtype

We examined the effects of a selective B_1-kinin receptor antagonist, des-Arg^9,[Leu^8]-bradykinin (Keravis *et al.*, 1991) and a selective B_2-kinin receptor antagonist, [Thi^{5,8}, D- Phe^7]-bradykinin (Keravis *et al.*, 1991) on endothelium-dependent relaxation. The B_1-kinin receptor antagonist (0.01 ≈ 1.0 μM)

Table 1. The effect of repeated application of bradykinin on the concentration-relaxation response of bovine coronary arteries with intact endothelium

Bradykinin	A type coronary arteries application				B type coronary arteries application			
-log M	1st	2nd	3rd	4th	1st	2nd	3rd	4th
15	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	7.1 ± 2.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
14	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	14.9 ± 7.5	0.8 ± 0.8*	0.0 ± 0.0*	0.0 ± 0.0*
13	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	17.3 ± 8.6	3.9 ± 3.5*	0.0 ± 0.0*	0.0 ± 0.0*
12	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 1.2	23.3 ± 10.0	8.2 ± 6.9*	0.0 ± 0.0*	0.8 ± 0.7*
11	14.1 ± 11.8	14.1 ± 10.4	0.1 ± 0.9	1.7 ± 4.0	34.1 ± 11.8	13.7 ± 11.1*	1.2 ± 0.9*	1.7 ± 2.0*
10	32.3 ± 12.2	25.7 ± 12.6	4.8 ± 3.2	16.5 ± 13.7	52.5 ± 11.8	25.9 ± 12.6*	8.8 ± 3.9*	16.5 ± 14.3*
9	68.9 ± 7.4	60.7 ± 9.2	51.8 ± 8.9	57.4 ± 12.2	74.4 ± 7.2	60.5 ± 9.6	51.8 ± 9.1	57.3 ± 12.4
8	80.5 ± 1.3	79.1 ± 1.2	75.1 ± 2.0	77.1 ± 3.9	81.4 ± 1.2	78.7 ± 1.3	74.9 ± 2.0	77.0 ± 3.9
7	86.0 ± 1.8	81.6 ± 3.1	78.8 ± 1.4	80.0 ± 2.8	86.0 ± 1.8	81.6 ± 3.1	78.7 ± 1.5	79.9 ± 2.8

The responses were obtained at intervals of about 120 min. Relaxation induced by 100 nM sodium nitroprusside was taken as 100%. Each point represents mean ± SEM ($n = 5$).*; Significantly different from the first response ($P < 0.05$).

had no significant effects ($P > 0.05$) on both types of relaxation ($n = 8$, each).

The B_2-receptor antagonist attenuated both types of relaxation in a concentration-dependent manner (Fig. 2). The calculated pA_2 value for the B_2-receptor (7.17 ± 0.10 in type A relaxation) was not significantly ($P > 0.05$) different from the pIC_{50} value (7.16 ± 0.65) for the B_2-receptor on the relaxant responses to 1 ≈100 fM bradykinin in type B relaxation.

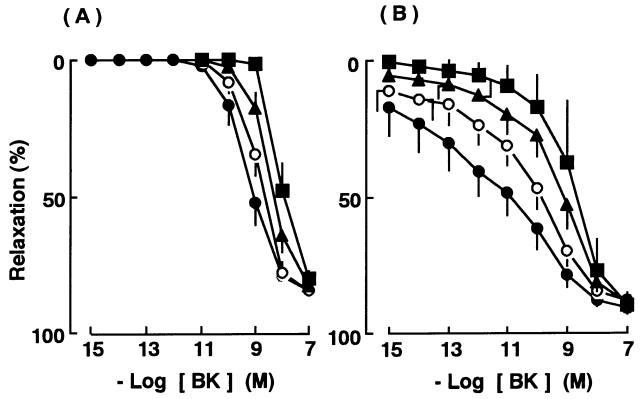

Fig. 2. Effect of [Thi^{5,8}, D-Phe^7]-bradykinin on concentration-response curves to bradykinin (BK) in bovine coronary arteries with intact endothelium (●; control, [Thi^{5,8}, D-Phe^7]-bradykinin 1 μM (○), 3 μM (▲), 10 μM (■)). Relaxation induced by 100 μM sodium nitroprusside was taken as 100%. Each point represents mean ± SEM ($n = 8$). (A), type A response; (B), type B response.

Effects of L-NAME and methylene blue

The effects of an NO synthase inhibitor, N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME; 100 μM), and a soluble guanylate cyclase inhibitor, methylene blue (10 μM) on type B relaxation were examined.

As shown in Fig. 3a, treatment with L-NAME or methylene blue significantly attenuated the relaxation, especially that induced by low concentrations of bradykinin. Inhibitory effects of L-NAME were significantly antagonized by treatment with L-, but not D-arginine (Fig. 3b).

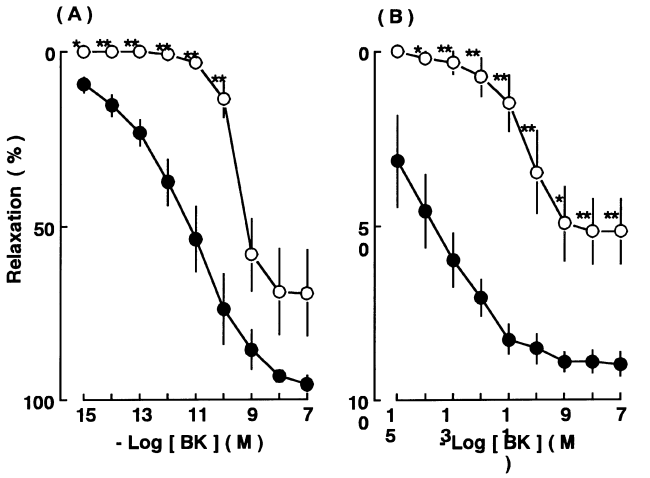

Fig. 3a. Effects of L-NAME or methylene blue (MB) on bradykinin (BK)-induced relaxtions in bovine coronary arteries with intact endothelium. Coronary rings were contracted with 3 μM $PGF_{2\alpha}$. Relaxation induced by 100 μM sodium nitroprusside was taken as 100%. Each point represents mean ± SEM ($n = 5$). (A) ●, control; ○, L-NAME (100 μM), (B) ●, control; ○, MB (10 μM). *, **; Significant differences from corresponding control values (*, $P < 0.05$; **, $P < 0.01$).

Effects of ACE inhibitors

Angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II and bradykinin to inactive peptides (Linz *et al.*, 1995). The difference between the type A and B relaxation responses to bradykinin might therefore depend on the distribution and activity of ACE. To examine this possibility, we examined the effects of two ACE inhibitors, captopril and enalapril, on bradykinin-induced relaxation. In the presence of captopril type A coronary arteries responded to fM bradykinin with relaxation, and fM ≈ 100 nM bradykinin-induced relaxations in type B arteries were potentiated (Fig. 4). In the presence of captopril, the type A and B responses were not significantly different. Similar results were obtained with enalapril (Fig. 4).

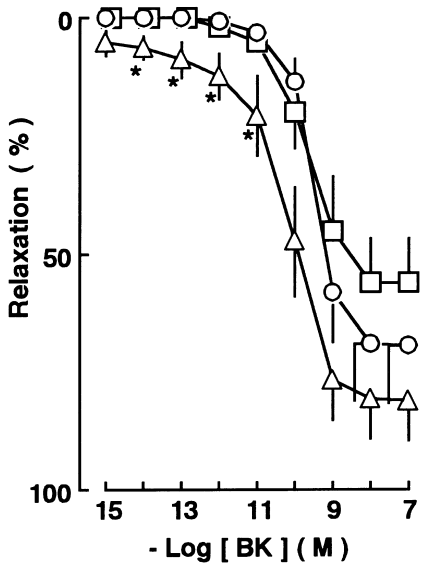

Fig. 3b. Effects of L- and D-arginine on the inhibitory effect of L-NAME. Coronary rings were treated either with 100 μM L-NAME only (○) or together with 9 mM L- (△) or D-arginine (□) for 30 min. Relaxation induced by 100 μM sodium nitroprusside was taken as 100%. Each point represents mean ± SEM ($n = 5$). *, Significant differences from corresponding value treated with L-NAME and L-NAME plus D-arginine ($P < 0.05$).

DISCUSSION

The present experiments show that some bovine coronary arteries respond to fM bradykinin by relaxation. This response appears to be mediated primarily by the release of nitric oxide via stimulation of the B_2-kinin receptor on the endothelium.

Relaxations induced by fM bradykinin disappeared when bradykinin was repeatedly applied. The desensitization induced by bradykinin in this experiment may be dependent on the receptor internalization suggested by Munoz & Leeb-Lundberg (1992) and Roscher *et al.* (1993). However, precise mechanisms are obscure at present.

Our previous report suggested that endothelium-dependent relaxation induced by nM bradykinin depends on the release of both nitric oxide and other endothelium-derived relaxing factor(s) (Obi *et al.*, 1993). In this experiment, however, relaxation induced by fM bradykinin was almost inhibited by treatment with a nitric oxide synthase inhibitor, L-NAME. This inhibition was significantly antagonized by simultaneous treatment with L-arginine but not with D-arginine, suggesting that relaxation induced by fM bradykinin depends largely on the production and release of nitric oxide from the endothelium. The inhibitory effect of methylene blue, a soluble guanylate cyclase inhibitor, strongly supports this suggestion.

The relaxation responses to fM and nM bradykinin were inhibited in a concentration-dependent manner by a B_2-kinin receptor antagonist, [Thi^{5,8}, D- Phe^{7}]-bradykinin. These results show that relaxation induced by fM bradykinin is mediated by the activation of endothelial B_2-kinin receptors. B_1-kinin receptors are thought to coexist with B_2-kinin receptors and are upregulated in certain pathological states (Bouthillier *et al.*, 1987; Bhoola *et al.*, 1992; Nakhostine *et al.*, 1993; Dewitt *et al.*, 1994; Brown *et al.*, 1995; Drummond & Cocks, 1995). A B_1-kinin receptor antagonist, des-Arg^{9}, [Leu^{8}]-bradykinin, however, did not affect relaxation induced by nM and fM bradykinin.

Why do only a small proportion of bovine coronary arteries respond to fM bradykinin? It has been reported that ACE inhibitors potentiate endothelium-dependent relaxation to bradykinin in bovine, canine and human coronary arteries by a local mechanism (Auch-Schwelk *et al.*, 1993; Zanzinger *et al.*, 1994; Bassenge, 1995; Hecker *et al.*, 1996). One can therefore speculate that either the distribution or activity of ACE, which also mediates bradykinin-breakdown, is less in those bovine coronary arteries responding to fM bradykinin. As shown in Fig. 4, ACE inhibitors markedly enhanced bradykinin-induced relaxation in both types of arteries. These results support the above speculation. Other possibilities are that ACE inhibitors may increase the affinity of the B_2-kinin receptor for bradykinin (Hecker *et al.*, 1996), and that bovine coronary endothelial cells may release endothelin, which counteracts nitric oxide-induced relaxation (Yanagisawa *et al.*, 1988; Ohde *et al.*, 1992; Kaito *et al.*, 1995; Zuccarello *et al.*, 1995). Later speculation is partly supported by the finding that ACE inhibitors inhibit endothelin release through B_2-kinin receptor-mediated mechanisms (Momose *et al.*, 1993). Further studies on these points are needed.

In conclusion, bovine coronary arteries show endothelium-dependent relaxation with fM bradykinin, which stimulates release of nitric oxide from the endothelium through stimulation

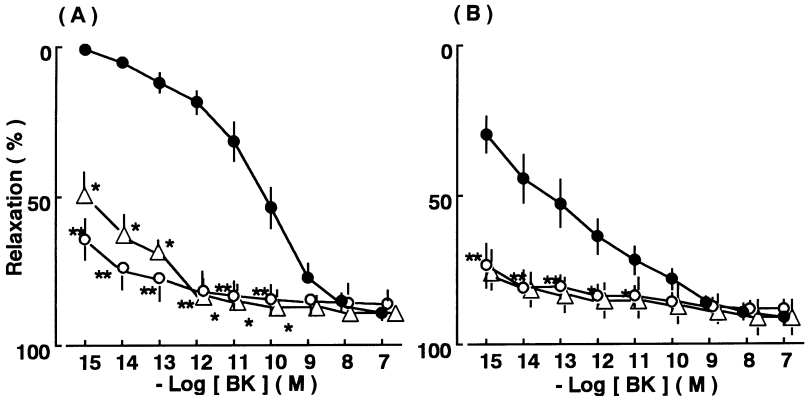

Fig. 4. Effects of captopril and enalapril on bradykinin (BK)-induced relaxations in bovine coronary arteries with intact endothelium. Coronary rings were partially contracted with 3 μM $PGF_{2\alpha}$. Relaxation induced by 100 μM sodium nitroprusside was taken as 100%. Each point represents mean ± SEM ($n = 10$). ●, control; ○, captopril (10 μM); △, enalapril (10 μM). (A), type A response; (B), type B response. *, **; Significant differences from corresponding control values (*, $P < 0.05$; **, $P < 0.01$).

of the B_2-kinin receptor. Relaxation induced by fM bradykinin might be regulated by ACE activity.

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