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Mechanism of contraction induced by bradykinin in the rabbit saphenous vein

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1 By using fura-PE3 fluorometry and receptor-coupled permeabilization by α-toxin, the mechanism of the bradykinin (BK)-induced contraction was determined in the rabbit saphenous vein (RSV). The receptor subtype responsible for the BK-induced contraction of RSV was determined by means of a pharmacological blocker study and reverse transcription polymerase chain reaction (RT-PCR).

2 In the presence of extracellular Ca^{2+} (1.25 mM), BK ($10^{-11}-3\times10^{-7}$ M) induced increases in both the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and force, in a concentration-dependent manner. Both the release of Ca^{2+} from the store site and the influx of extracellular Ca^{2+} contribute to an increase in $[Ca^{2+}]_i$ induced by BK.

3 In the absence of extracellular Ca^{2+}, the application of 10^{-7} M BK induced transient elevations of $[Ca^{2+}]_i$ and force, both of which thereafter declined to the levels observed before the application of BK. When extracellular Ca^{2+} was replenished (1.25 mM), $[Ca^{2+}]_i$ and force increased to form a peak, followed by a sustained elevation in the presence of BK. When an RSV strip was pretreated with 10^{-5} M thapsigargin for 20 min, the BK-induced transient increases in both $[Ca^{2+}]_i$ and force were markedly inhibited.

4 These responses induced by BK were inhibited by Hoe 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8] bradykinin), a highly specific bradykinin B_2 receptor antagonist, in a concentration-dependent manner. In RT-PCR, B_2-receptor mRNA was expressed in the smooth muscle of RSV.

5 The $[Ca^{2+}]_i$-force relationships, which were determined by cumulative applications of extracellular Ca^{2+} (0–5 mM) during 118 mM K^+-depolarization, shifted to the upper left in the presence of BK, thus indicating that BK induced a greater force than 118 mM K^+-depolarization for a given level of $[Ca^{2+}]_i$.

6 In α-toxin-permeabilized preparations of RSV, application of 10^{-7} M BK after a steady state contraction had been induced by a mixture of 5×10^{-7} M Ca^{2+}, 10^{-6} M GTP and 10^{-6} M captopril caused an additional force development at a constant $[Ca^{2+}]_i$. However, treatment with 1 mM guanosine-5′-*O*-(β-thiodiphosphate) (GDPβS) for 5 min before and during the application of BK (10^{-7} M), abolished this BK-induced additional contraction.

7 These results indicated that in RSV: (1) BK elicits vasoconstriction by increasing the Ca^{2+} influx from the extracellular space, Ca^{2+} release from intracellular thapsigargin-sensitive storage sites and increasing the Ca^{2+} sensitivity of the contractile apparatus, (2) the BK-induced increase in Ca^{2+} sensitivity is mediated by G-protein, (3) the BK-induced contractions are mediated via B_2-receptors and (4) the smooth muscle cells express B_2-receptor mRNA.

Keywords: Bradykinin; B2 receptor; mRNA; vascular smooth muscle; cytosolic calcium concentration; thapsigargin; saphenous vein

Introduction

Bradykinin (BK) is a potent vasoactive peptide as well as a mediator of pain, inflammation, fluid balance, vascular permeability and gastrointestinal function (Regoli & Barabe, 1980; Steranka *et al*., 1988 Roberts, 1989; Kingden-Milles, 1995). Since the application of BK *in vivo* induces vasodilatation and hypotension in animals (Regoli & Barabe, 1980; Browning *et al*., 1987; Regoli *et al*., 1989) and man (Bonner *et al*., 1990; 1992; Dachman *et al*., 1993), the major role of BK on vascular smooth muscle has been thought to be an endothelium-dependent vasodilatation. Therefore, in relation to vasorelaxation, the effects of BK on endothelial cells and arterial strips with intact endothelium have been extensively investigated (Lambert *et al*., 1986; Mombouli *et al*., 1992; Hecker *et al*., 1992; Nakashima *et al*., 1993; Freming *et al*., 1994; Blatter *et al*., 1995). On the other hand, BK also induces contractions of venous preparations, such as the guinea-pig anterior mesenteric vein (Gaudreau *et al*., 1981), the rat portal vein (Regoli *et al*., 1994), the rabbit jugular vein (Calixto & Medeiros, 1992), the rabbit vena cava (Nantel *et al*., 1991; Regoli *et al*., 1994) and the rabbit mesenteric vein (Regoli *et al*., 1977). The vasoconstrictive effect of BK on veins may play an important physiological role *in vivo*, because an increase in venous pressure may lead to an increase in vascular permeability, that is efflux of the fluid from the blood vessels to tissue (Whalley *et al*., 1987). However, the mechanisms for the BK-induced contraction of the venous smooth muscle cells have not yet been fully elucidated.

Although it is generally accepted that the contraction of the smooth muscle is primarily regulated by $[Ca^{2+}]_i$ (Kamm & Stull, 1985), it has been shown that smooth muscle contractile force does not depend on $[Ca^{2+}]_i$ alone (Morgan & Morgan, 1984). It was found that agonist-stimulation of the smooth muscle induced an increase in Ca^{2+} sensitivity of the contractile apparatus (Nishimura *et al*., 1988; Kitazawa *et al*., 1989; Kobayashi *et al*., 1989). It has also been shown that the BK increases $[Ca^{2+}]_i$ in the cultured vascular smooth muscle

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cells (Dixon *et al*., 1990; Briner *et al*., 1993). However, there have been no previous studies which show the effects of BK on the $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of intact vascular smooth muscle strips.

Regoli & Barabe (1980) suggested that BK and related kinins may act on two different receptor subtypes, named B_1 and B_2 (Regoli & Barabe, 1980). It has been postulated that B_2 receptors are a constitutive type, while B_1 are an inducible type of receptor (Regoli *et al*., 1978). The development of specific, sequence-related peptide antagonists for B_1- and B_2-receptors revealed that the contractions of the vascular smooth muscle are mediated by both B_1- and B_2-subtypes. Recently, cDNA cloning of B_1-receptors was performed from the mRNA of the rabbit aorta (MacNeil *et al*., 1995). However, the expression of B_2-receptors in vascular smooth muscle cells based on both functional and molecular biological studies has not yet been investigated, although the molecular cloning of B_2-receptors has already been done in man (Hess *et al*., 1992), rats (McEachern *et al*., 1991), mice (Ma *et al*., 1994) and rabbits (Bachrov *et al*., 1995).

In the present study, we investigated the mechanisms underlying the BK-induced contraction of the rabbit saphenous vein (RSV) by use of front-surface fluorometry with fura-PE3 and receptor-coupled permeabilization by a α-toxin. The receptor subtype responsible for the BK-induced contraction of RSV was also determined based on both a pharmacological blocker study and the expression of B_2-receptor mRNA. In this study, for the first time, we present evidence that BK acts on B_2-receptors and induces contraction by increasing $[Ca^{2+}]_i$ and the Ca^{2+} sensitivity of the contractile apparatus through the activation of G-protein in RSV.

Methods

Tissue preparation

The study protocol was approved by the Animal Care and Committee of Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University. Japanese white rabbits (male, 16–20 weeks old, body weight 2.5–3.0 kg) were killed by sodium pentobarbitone (100 mg kg^{-1}, i.v.) and then the lateral saphenous veins (RSV) were immediately excised. The adventitia and fat were removed by dissection, under a binocular microscope. The endothelial cells were then removed by rubbing the intraluminal surface with a cotton swab (Furchgott & Zawadzki, 1980). Medical preparations were cut into approximately 1.5×3 mm circular strips, 0.2 mm thick.

Fura-PE3 loading

The medial strips of RSV thus obtained were loaded with fura-PE3 in the form of acetoxymethyl ester (fura-PE3/AM) by incubation in Dulbecco's modified Eagle's medium containing 40 μM fura-PE3/AM, 5% foetal bovine serum and 0.1% Pluronic F-127 for 6 h at 37°C. Subsequently, the strips were washed with physiological salt solution (PSS) containing 1.25 mM Ca^{2+} at 37°C to remove the dye from the extracellular space and were then equilibrated in PSS for at least 1 h before the initiation of measurements. The strips thus treated showed a specific peak of the fluorescence emission spectrum for fura-PE3 (500 nm) as well as a specific peak and valley of the fluorescence excitation spectrum for fura-PE3, 340 nm and 380 nm, respectively, which were determined by a fluorescence spectrophotometer (model 650-40, Hitachi, Tokyo, Japan). Loading the vascular strips with fura-PE3 did not alter the time course or the maximum levels of force development during the 118 mM K^+-induced depolarization (data not shown), which suggested that no tissue damage occurred due to any possible acidification of the cells, as a result of formaldehyde release on AM-ester hydrolysis (Tsien *et al*., 1982; Rink & Pozzan, 1985).

Measurement of force

RSV strips were mounted vertically in a quartz organ bath, and the isometric force development was measured by a force-transducer (strain gauge TB-612T, Nihon Kohden, Japan). During the 1 h equilibration period, the strips were stimulated with 118 mM K^+ every 15 min, and the resting load was increased in a stepwise manner to obtain the maximal force development. The resting load for the measurements, thus obtained, was between 200 and 250 mg. The force development was expressed as a percentage; the values in normal (5.9 mM K^+) and 118 mM K^+PSS were assigned to be 0% and 100%, respectively.

Measurement of $[Ca^{2+}]_i$

Changes in the fluorescence intensity of the fura-PE3-Ca^{2+} complex were simultaneously monitored during the measurement of the force, by use of front-surface fluorometry (Abe *et al*., 1990; Hirano *et al*., 1991; Ushio-Fukai *et al*., 1993). The fluorescence intensity was measured by a fluorometer, which was specially designed for fura-2 fluorometry (CAM-OF-1). In brief, the strips were illuminated by guiding the alternating (400 Hz) 340 nm and 380 nm excitation light from a xenon light source through quartz optic fibres arranged in a concentric inner circle (diameter = 3 mm). The surface fluorescence of the strips was collected by glass optic fibres arranged in an outer circle (diameter = 7 mm) and introduced through a 500 nm band-pass filter (full width at half maximum transmission = 10 nm) into a photon-counting photomultiplier. The data of 500 nm fluorescence intensities at 340 nm (F340) and 380 nm (F380) excitation, fluorescence ratio (F340/F380) and force were stored in a Macintosh computer by a data acquisition system (MacLab) with a sampling rate of 17 Hz. The fluorescence ratio (F340/F380), which indicates $[Ca^{2+}]_i$, was expressed as a percentage, assigning the values in normal (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively, because these % values were stable during the time course of measurements in the present study. For the determination of the EC_{50} value of BK for the increase in $[Ca^{2+}]_i$, the absolute values of $[Ca^{2+}]_i$ were determined in RSV strips in a seperate experiment as described by Grynkiewicz *et al*. (1985). The values of $[Ca^{2+}]_i$ at rest (0%) and during 118 mM K^+-depolarization (100%) obtained from 8 separate samples were 181 ± 22 nM and 881 ± 98 nM, respectively.

Experimental protocols for the simultaneous determination of $[Ca^{2+}]_i$ and force in the BK-induced contraction

All the measurements were performed at 37°C. Before each measurement, the resting levels (0%) and the response levels (100%) to 118 mM K^+ depolarization in PSS were recorded. Ten minutes before the application of BK, 10^{-6} M captopril was applied to prevent the degradation of BK by kininase II (Regoli & Barabe, 1980).

Permeabilization by α-toxin

Permeabilization of RSV was performed as previously described (Nishimura *et al*., 1988) with minor modifications. Briefly, small rings from the segments of RSVs were dissected and trimmed. Two tungsten wires were passed through the lumen. One wire was fixed to the chamber while the other was attached to a force transducer (U gage, Minebea Co. Ltd., Japan). Permeabilization was accomplished by incubating the venous segments with *Staphylococcal* α-toxin (5000 unit ml^{-1}, Calbiochem, La Jolla, CA) for 60 min in a Ca^{2+}-free cytoplasmic substitution solution (CSS) containing 2 mM EGTA. The apparent binding constant used for the Ca^{2+}-EGTA was 10^6/M (Saida & Nonomura, 1978). After permeabilization, the tissue specimens were stretched by a micromanipulator connected to the force transducer, to the appropriate resting load

which gave the maximum contraction by 10^{-5} M Ca^{2+} containing CSS solution. The force development in the α-toxin permeabilized tissue was determined at room temperature. Forces in Ca^{2+}-free CSS containing 2 mM EGTA and those induced by 10^{-5} M Ca^{2+} were designated to be 0 and 100%, respectively.

Measurement of B_2-receptor mRNA by reverse transcription (RT) polymerase chain reaction (PCR)

Total RNA was isolated from the smooth muscle cells from RSV according to the method described by Chomczynksi & Sacchi (1987). To obtain the smooth muscle cells, care was taken not to include either the endothelium or adventitia during the tissue trimming, as shown in the tissue preparation section. Contaminating genomic DNA, if any, was digested by RNase free DNase. First-strand cDNA was synthesized with the total RNA for the template. The total RNA (1 μg) was incubated at 37°C with a mixture (total volume = 20 μl) of 200 units of M-MLV reverse transcriptase, 1 × RT buffer, 10 mM DTT, 0.5 mM each dNTPs (dATP, dCTP, dTTP, dGTP), 20 units of RNase inhibitor and 50 nM of antisense primers. For the PCR amplification, an aliquot (1 μl) of RT product was mixed with 0.5 unit of Taq DNA polymerase, 500 nM each of sense and antisense primers in a buffer containing 20 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg ml^{-1} BSA and 200 μM each of dNTPs in a 11 μl volume. The thermal cycle profile used in this study was (1) denaturing for 30 at 94°C, (2) annealing primers for 90 at 55°C and (3) extending the primers for 30 at 72°C. PCR amplification was performed for 45 cycles. The sequences of sense and antisense primers were designed based on the published sequence for rabbit B_2-receptors (Bachrov *et al*., 1995). Primers for PCR were 5′-TCA CCA TCG CCA ACC ACT TC-3′ (sense: 272-292) and 5′-GTA GCC CTC GTC TCG GTA GT-3′ (antisense: 527–547), and primer for RT was 5′-AGC CAG CAC ACC ACG AAC AG-3′ (antisense: 760–780). The expected size of the PCR product for B_2-receptors was 275 bp. A portion (10 μl) of the PCR mixture was electrophoresed in a 3% agarose gel in TAE buffer. The gel was stained with ethidium bromide and then photographed.

Drugs and solutions

The physiological salt solution (PSS) was of the following composition (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25, and D-glucose 11.5. High K^+ PSS was identical to PSS, except for an equimolar substitution of KCl for NaCl. The Ca^{2+}-free solution was identical to PSS, except for a substitution of 2 mM ethyleneglycol-*bis*(P-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA) for 1.25 mM $CaCl_2$. All solutions were gassed with a mixture of 95% O_2 and 5% CO_2 (pH adjusted to 7.4 at 37°C).

The composition of the cytoplasmic substitution solution (CSS) was (in mM): EGTA 10, K-methanesulphonate 100, $MgCl_2$, 3.38, Na_2ATP 2.2, creatine phosphate 10, Tris maleate (pH = 6.8) 20, and the indicated concentration of free Ca^{2+}. Bradykinin (BK) was obtained from the Peptide Institute Co. Ltd. (Osaka, Japan). Fura-PE3/AM was from the Texas Fluorescence Laboratory (Austine, TX, U.S.A.). Pluronic F-127 was from the BASF Co. (Parsippany, NJ, U.S.A.) Bovine serum albumin (BSA) and thapsigargin were from Sigma (St. Louis, MO, U.S.A.). Hoe 140 (D-Arg-[Hyp^3, Thi^5, D,-Tic^7, Oic^8] bradykinin) was kindly donated by Hoechst AG (Frankfurt, FRG). Captopril was kindly donated by the Sankyo Co. (Tokyo, Japan). M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase, 5 × RT buffer and 0.1 M DTT (dithiothreitol) were from BRL (Gaithersburg, MD, U.S.A.). Nusieve 3:1 agarose and dNTPs (dATP, dCTP, dTTP, dGTP) were from TaKaRa (Kyoto, Japan). RNase inhibitor was purchased form Toyobo (Osaka, Japan). Taq DNA polymerase was from Pharmacia Biotech (Uppsala, Sweden). RQ1 RNase-free DNase was from Promega (Madison, WI, U.S.A.). Rsa I was purchased from Wako (Osaka, Japan). All other chemicals were of the highest grade commercially available. The oligonucleotides for primers were synthesized by Sawady Technology (Tokyo, Japan).

Data analysis

All data for the simultaneous measurements of $[Ca^{2+}]_i$ and force were collected by a computerized data acquisition system (MacLab, Analog Digital instruments, Castle Hill, Australia: Macintosh, Apple Computer, Cupertino, CA, U.S.A.). The data for the representative traces shown in this study were directly printed from the computer to a laser printer (LaserWriter II NTX-J, Apple Computer). The measured values are expressed as the means ± s.e. (n = number of the experiments). For each experiment, a strip from a different animal ($n = 5–9$) was used. For comparison, unpaired Student's t test was used and P values of less than 0.05 were considered to be significant. Statistical analysis of the $[Ca^{2+}]_i$-force curves was carried out by analysis of covariance.

Results

Effect of BK on $[Ca^{2+}]_i$ and force in RSV strips in PSS

When the RSV strip was exposed to 10^{-7} M BK in PSS, $[Ca^{2+}]_i$ and force rose rapidly and reached a peak (the first phase) in 1–3 min, and then slowly declined, but remained at levels higher than those prestimulation (the second phase). The BK (10^{-7} M)-induced increases in $[Ca^{2+}]_i$ and force at the first phase were 104 ± 3% and 144 ± 8% ($n = 6$), respectively, while those at the second phase (15 min after the application) were 80 ± 8% ($[Ca^{2+}]_i$) and 115 ± 11% (force: $n = 6$) of those observed during 118 mM K^+-depolarization. As shown in Figure 1, cumulative applications of BK (10^{-11}-3×10^{-7} M) led to step-wise, concentration-dependent ($P < 0.01$, by an analysis of variance, $n = 5$) increases in $[Ca^{2+}]_i$ and force. The EC_{50} values for increases in $[Ca^{2+}]_i$ and force were $(2.4 \pm 1.5) \times 10^{-9}$ and $(1.7 \pm 0.5) \times 10^{-9}$, respectively. There was no significant difference between these two values.

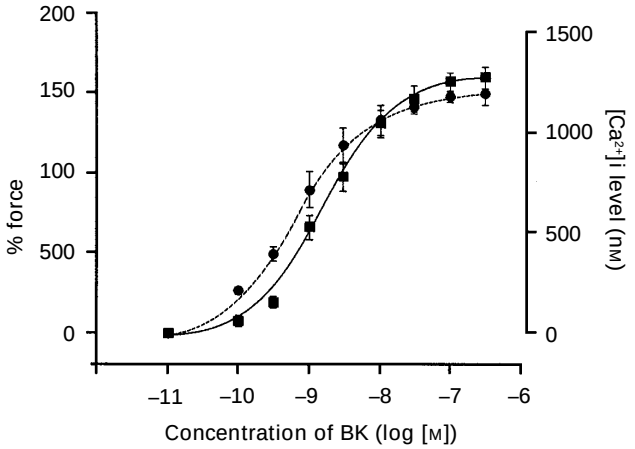

Figure 1 The concentration-response curves for BK. BK (10^{-11}-3×10^{-7} M) induced increases in $[Ca^{2+}]_i$ (●) and force (■) in a concentration-dependent manner. EC_{50} values for BK for the increases in $[Ca^{2+}]_i$ and force were $(2.4 \pm 1.5) \times 10^{-9}$ M and $(1.7 \pm 0.5) \times 10^{-9}$ M, respectively. Absolute values of $[Ca^{2+}]_i$ were determined by using Grynkiewicz's equation (1985). As shown in the Methods, $[Ca^{2+}]_i$ at rest (0%) and during 118 mM K^+-depolarization (100%) were 181 ± 22 nM and 881 ± 98 nM, respectively. All values are the means and vertical lines show s.e.mean ($n = 5$).

BK-induced Ca^{2+} transients in Ca^{2+}-free PSS and effect of thapsigargin

As shown in Figure 2a, when the strip was exposed to Ca^{2+}-free PSS (Ca^{2+}-free PSS containing 2 mM EGTA for 5 min, and then, Ca^{2+}-free PSS, $[Ca^{2+}]_i$ gradually decreased to reach a new steady level after about 10 min. Application of 10^{-7} M BK during this steady state induced transient increases in $[Ca^{2+}]_i$ and force. This intracellular Ca^{2+}-dependent elevation of $[Ca^{2+}]_i$ was considered to be caused by BK-activated Ca^{2+}-release from the store site. The subsequent restoration of ex-

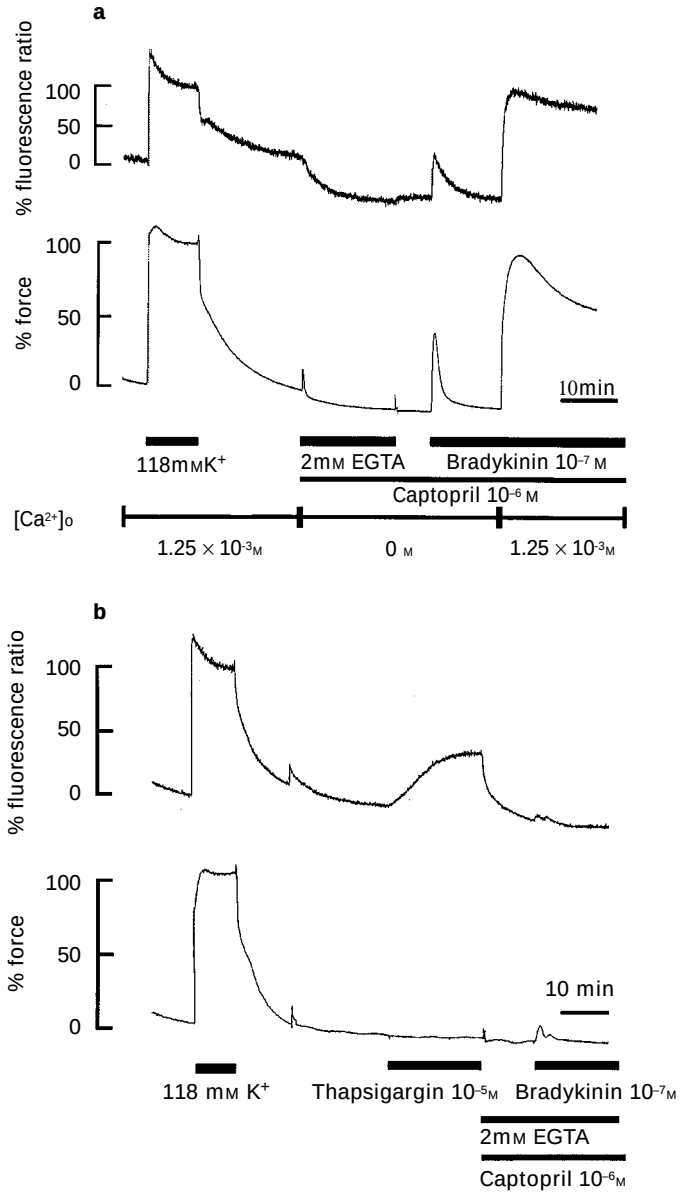

Figure 2 Representative recordings of the effects of BK on $[Ca^{2+}]_i$ and force of RSV in Ca^{2+}-free PSS. (a) Representative recordings of $[Ca^{2+}]_i$ (upper trace) and force (lower trace) during restoration of extracellular Ca^{2+} after exposure of RSV strips to Ca^{2+}-free PSS in the presence of BK. Application of BK in the Ca^{2+}-free PSS induced transient increases in $[Ca^{2+}]_i$ and force. The restoration of extracellular Ca^{2+} in the presence of 10^{-7} M BK caused a rapid elevation of $[Ca^{2+}]_i$ and tension which thereafter gradually declined, but remained at a sustained elevated level. (b) The effect of thapsigargin (10^{-5} M) on the transient increases in $[Ca^{2+}]_i$ (upper trace) and force (lower trace) induced by BK (10^{-7}M) in the absence of extracellular Ca^{2+}. Preincubation for 20 min with thapsigargin markedly inhibited the transient increases in $[Ca^{2+}]_i$ and force induced by BK in the absence of extracellular Ca^{2+}.

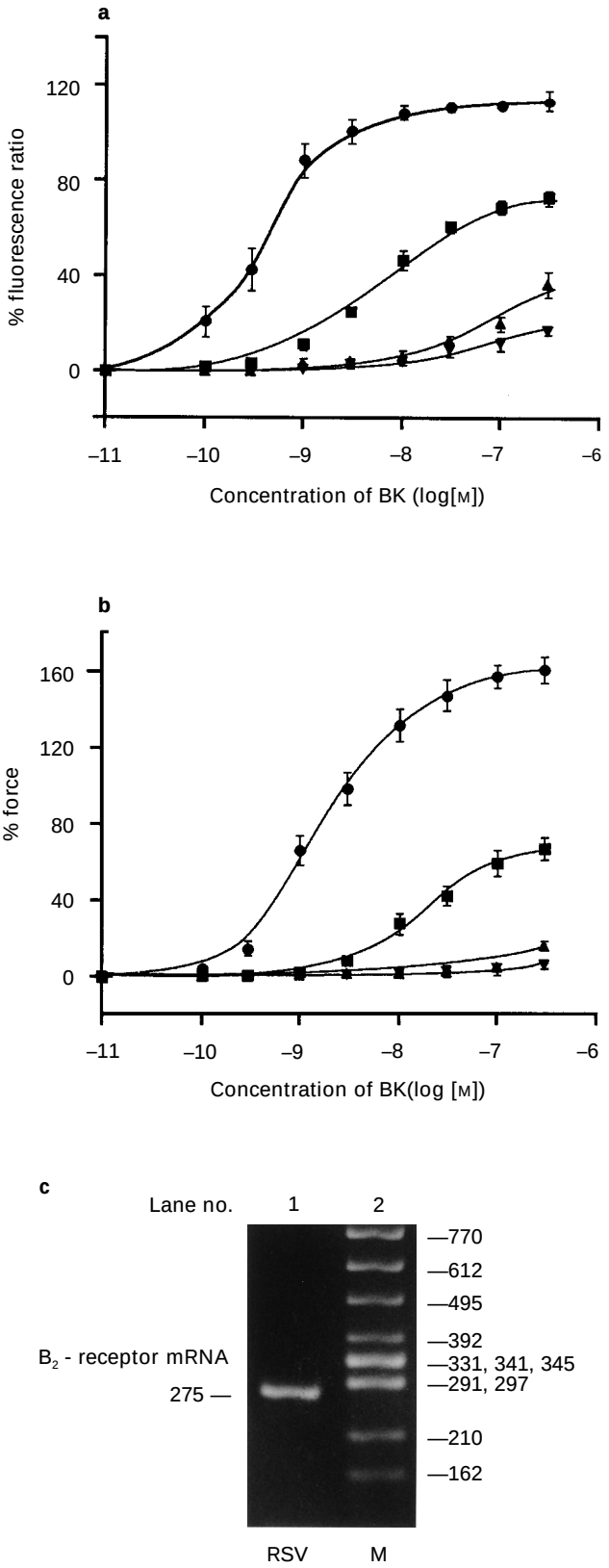

Figure 3 (a and b) The inhibitory effect of Hoe140 on $[Ca^{2+}]_i$ (a) and force (b) at the steady state of the contraction induced by BK ($10^{-11}-3\times10^{-7}$ M). Hoe140 (●, 0 M; ■, 10^{-10} M ▲, 10^{-9} M; ▼ 10^{-8} M) inhibited BK-induced increases in $[Ca^{2+}]_i$ and force in a concentration-dependent manner. All values are the means and vertical lines show s.e.mean ($n=5$). (c) Detection of B_2-receptor mRNA in RSV by RT-PCR. PCR amplification for B_2-receptors were performed 45 cycles. The predicted size of the PCR amplification products is shown on the left of the photograph. Lane 1 represents the PCR product for the B_2-receptor. Lane 2 represents the DNA size marker (M;ØX174/Hinc II digest). The size of each band is shown on the right side of the photograph.

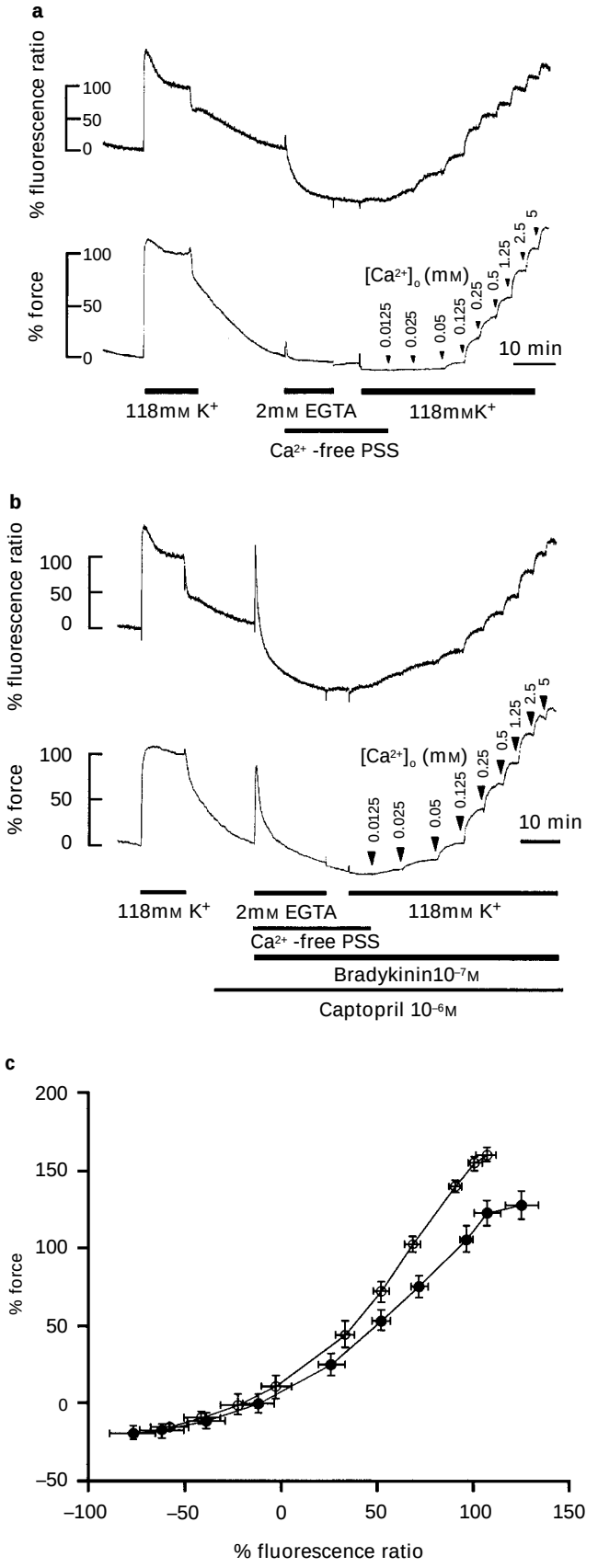

Figure 4 Representative recordings of the changes in $[Ca^{2+}]_i$ (upper trace) and force (lower trace) induced by the cumulative applications of extracellular Ca^{2+} (0–5 mM) during 118 mM K^+-depolarization in the absence (a) and the presence of BK (b). The extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) is shown in the lower panel. Both $[Ca^{2+}]_i$ and force increased stepwise with elevations of $[Ca^{2+}]_o$. The steady-state $[Ca^{2+}]_i$-tension curves (c) in the absence (●) or presence of BK (○) were constructed with the data obtained from 9 independent experiments done in the same manner as in (a) and (b). The data were obtained at the time that force reached the maximal level at each application of extracellular Ca^{2+}. The vertical and horizontal bars represent s.e.mean.

tracellular Ca^{2+} to 1.25 mM in the presence of BK induced rapid increases in $[Ca^{2+}]_i$ and force to form peaks and subsequent sustained elevation. Since this sustained elevation of $[Ca^{2+}]_i$ was not observed during the restoration of extracellular Ca^{2+} in the absence of BK following the exposure of strips to Ca^{2+}-free PSS (data not shown), this extracellular Ca^{2+}-dependent elevation was thus considered to be caused by BK-activated Ca^{2+}-influx. When the vascular strip was treated with 10^{-5} M thapsigargin for 20 min in PSS, $[Ca^{2+}]_i$ gradually increased to reach a steady state (46±5.0%) without any force development (Figure 2b). After the treatment with thapsigargin, the BK-induced transient increases in $[Ca^{2+}]_i$ and force in the Ca^{2+}-free PSS were markedly inhibited, as shown in Figure 2b.

BK receptor characterization by using an antagonist and RT-PCR

Hoe140, a new highly selective B_2 antagonist (Hock *et al*., 1991; Rhaleb *et al*., 1992), inhibited the increases in $[Ca^{2+}]_i$ (Figure 3a) and force (Figure 3b) induced by BK (10^{-11} M–3×10^{-7} M) in a concentration-dependent manner (10^{-10} M–10^{-8} M) ($P<0.05$ for Hoe140, by a two way analysis of variance, $n=5$). Hoe140 induced not only a rightward shift but also a downward shift of the concentration-response curves. As shown in Figure 3c, the B_2-receptor bands of the expected size (275 bp) could be detected by RT-PCR, by total RNA prepared from smooth muscle of RSV and specific primers for the rabbit B_2-receptor. The PCR products for B_2-receptors from RSV could be digested by Rsa I into the fragments of the predicted size in agarose gel electrophoresis (data not shown).

$[Ca^{2+}]_i$-force relationship during contractions induced by BK

Figure 4a shows representative recordings of the changes in $[Ca^{2+}]_i$ and force induced by cumulative applications of external Ca^{2+} (0–5 mM) during 118 mM K^+-depolarization. After the responses (100%) to 118 mM K^+-depolarization had been recorded, the vascular strips were incubated in Ca^{2+}-free PSS containing 2 mM EGTA for 10 min, followed by a 5 min exposure to Ca^{2+}-free PSS without EGTA. Then the solution was changed to a Ca^{2+}-free 118 mM K^+-depolarization solution and extracellular Ca^{2+} (0–5 mM) was cumulatively applied. Both $[Ca^{2+}]_i$ and the force increased stepwise with elevations of the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). The fluorescence ratio increased from −77±12% (at 0 M $[Ca^{2+}]_o$; $n=9$) to 125±9% (at 5 mM $[Ca^{2+}]_o$; $n=9$). The force increased from −19±4% to 128±9% ($n=9$). Figure 4b shows representative recordings of the cumulative application of external Ca^{2+} during 118 mM K^+-depolarization in the presence of 10^{-7} M BK. The fluorescence ratio increased from −58±10% (at 0 M $[Ca^{2+}]_o$; $n=9$) to 106±5% (at 5 mM $[Ca^{2+}]_o$; $n=9$). The force increased from −15±2% to 161±5% ($n=9$). In Figure 4c, the $[Ca^{2+}]_i$-force relationships were plotted with the data points obtained from the experiments carried out in the same manner as described in Figure 4a and b. BK (10^{-7} M) shifted the $[Ca^{2+}]_i$-force curve to the upper left ($P<0.002$, by analysis of covariance), indicating that BK induces a greater force for the given levels of $[Ca^{2+}]_i$.

The effects of BK on the tension development in α-toxin-permeabilized preparations

As shown in Figure 5a, application of 10^{-7} M BK, after a steady state contraction had been induced by a mixture of

5×10^{-7} M Ca^{2+}, 10^{-6} M guanosine 5′-triphosphate (GTP) and 10^{-6} M captopril, caused additional force development at a constant $[Ca^{2+}]_i$. Treatment with 1 mM guanosine-5′-*O*-(*β*-thiodiphosphate) (GDP*β*S) for 5 min before and during the application of BK (10^{-7} M) abolished this BK-induced additional force development (Figure 5b).

Discussion

The major findings of the present study are (1) BK increases the Ca^{2+} sensitivity of the contractile apparatus, which is mediated by the activation of G-protein, (2) BK elevates $[Ca^{2+}]_i$ of RSV as a result of the Ca^{2+} influx from the extracellular space and Ca^{2+} release from the intracellular store, and (3) The receptors responsible for the BK-induced contraction of RSV are the B_2 subtype, determined by both the pharmacological blocker study and RT-PCR.

The conclusion that BK increases the Ca^{2+} sensitivity of the contractile apparatus mediated by the activation of G-protein in RSV was based on the following observations. As shown in Figure 4, for a given steady-state level of $[Ca^{2+}]_i$, force development in the presence of BK was greater than that in the absence of BK. In the receptor coupled permeabilized preparation, BK enhanced the contraction at a given $[Ca^{2+}]_i$ in the presence of GTP, as shown in Figure 5a. The BK-induced enhancement of force was blocked by the presence of GDPβS, thus indicating the involvement of G protein in this increase in the Ca^{2+} sensitivity (Figure 5b). The activation of G-protein is one of the known mechanisms of increases in Ca^{2+} sensitivity of vascular smooth muscle (Nishimura *et al*., 1988; Kitazawa *et al*., 1989; Kobayashi *et al*., 1989). Therefore, our present data provide another example for the coupling of G protein being involved in agonist (BK)-induced increases in Ca^{2+} sensitivity of the contractile apparatus. The site of action of GTP for the increase in Ca^{2+} sensitivity of the contractile apparatus of the smooth muscle is still unclear. It was initially thought to be the heterotrimeric G protein coupled with the receptors (Kitazawa *et al*., 1989). However, recent research has indicated that small molecular weight G proteins, rho A p21, might be involved in the increase in Ca^{2+} sensitivity of the contractile apparatus. Hirata *et al*. (1992) showed that EDIN, a specific inhibitor of rho A p21, inhibited the contraction induced by GTPγS of permeabilized vascular smooth muscle strips, and that this inhibition could be recovered by the addition of GTPγS-stimulated rho A p21. Therefore, the site of action of GTP might be located on the large and/or small G proteins.

The precise intracellular mechanism for the increase in Ca^{2+} sensitivity of the contractile apparatus in smooth muscle is currently unknown. At least two mechanisms have been proposed with the permeabilized preparation; one is a myosin light chain (MLC) phosphorylation dependent mechanism (Kitazawa *et al*., 1991; Moreland *et al*., 1992) and the other is a MLC phosphorylation-independent one (Moreland *et al*., 1992). For the former mechanism, it has been proposed that the increase in Ca^{2+} sensitivity involves the inhibition of MLC phosphatase (Kitazawa *et al*., 1991). Very recently, Kimura *et al*. (1996) found that MLC phosphatase could be inhibited by the activation of a small G protein, rho A p21, and subsequent activation of rho associated kinase. It remains to be clarified as to whether the BK-induced increases in Ca^{2+} sensitivity might involve these pathways.

In the presence of extracellular Ca^{2+}, BK-induced increases in $[Ca^{2+}]_i$ and force in a concentration-dependent manner (Figure 1). The elevation of $[Ca^{2+}]_i$ induced by BK consisted of two components. The first phase was composed of extracellular Ca^{2+}-independent and dependent components, while the second phase was exclusively extracellular Ca^{2+}-dependent, because the first phase became much smaller and transient and the second phase was abolished in the absence of extracellular Ca^{2+} (Figure 2a). Therefore, the second phase and part of the first phase of $[Ca^{2+}]_i$ elevations were attributed

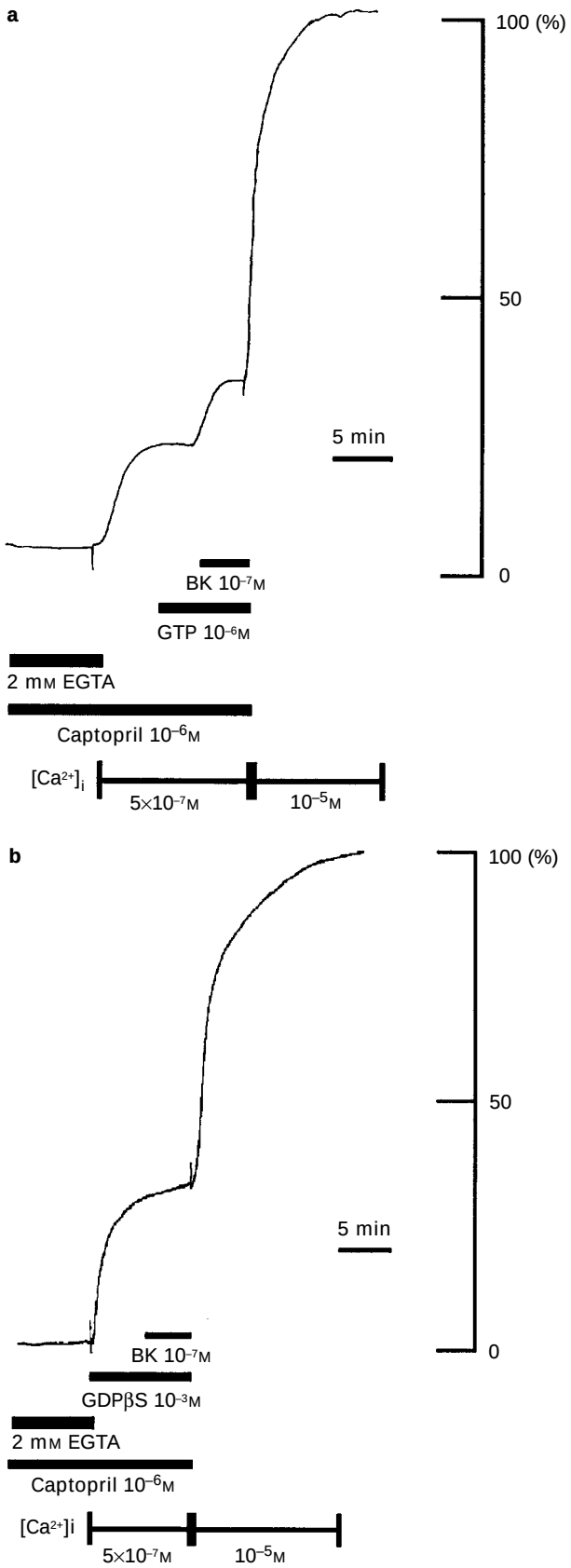

Figure 5 Effect of guanine nucleotides on the contractile responses induced by BK in α-toxin-permeabilized RSV. (a) The application of 10^{-7} M BK in the presence of 10^{-6} M GTP induced contraction in the highly buffered Ca^{2+} (5×10^{-7} M buffered by 10 mM EGTA) solution. (b) pretreatment with 1 mM GDP*β*S abolished the contraction induced by 10^{-7} M BK. In (a) and (b), strips were treated with 10^{-6} M captopril 10 min before and during BK application.

to the influx of extracellular Ca^{2+}. The $[Ca^{2+}]_i$ elevation induced by 10^{-7} M BK could be inhibited only partially by diltiazem and verapamil (data not shown), thus indicating that BK-induced Ca^{2+} influx may be mainly through channels other than voltage-operated Ca^{2+} channels. This speculation is in good agreement with previous results obtained by other investigators (Nantel *et al*., 1991; Calixto & Medeiros, 1992). However, the type of Ca^{2+} channel activated by BK remains to be clarified.

The extracellular Ca^{2+}-independent component of the first phase was possibly due to the release of Ca^{2+} from the intracellular store sites, presumably sarcoplasmic reticulum (SR), because this component was abolished by thapsigargin, an SR Ca^{2+} pump inhibitor (Figure 2b). The present study also showed that the response of RSV to BK is mediated by G protein. These results are consistent with the idea that BK receptor (B_2 subtype) signal transduction in RSV involves G protein-mediated breakdown of phosphatidylinositol catalysed by phospholipase C, as in the case of other Ca^{2+} mobilizing receptors (Dixon *et al*., 1990; Calixto & Medeiros, 1992; Briner *et al*., 1993). In addition, as shown in Figure 2b, treatment of RSV strip with thapsigargin in PSS induced a gradual increase in $[Ca^{2+}]_i$ without any force development. This phenomenon could be explained by the so-called 'capacitative model' theory, in which the influx of extracellular Ca^{2+} is controlled by the filling state of intracellular Ca^{2+} stores (Putney, 1990). This phenomenon was also documented in smooth muscle strips (Uyama *et al*., 1993; Higuchi *et al*., 1996). The inability of the SR Ca^{2+} pump inhibitor, thapsigargin, to induce contraction despite an increase in $[Ca^{2+}]_i$ ($46 \pm 5.0\%$) could be explained by its inability to increase Ca^{2+} sensitivity of the contractile apparatus.

In the present study, we provide evidence that functioning B_2-receptors and their mRNA are present in smooth muscle cells of RSV. As shown in Figure 3a and b, Hoe140, a potent selective antagonist of B_2-receptors (Hock *et al*., 1991), inhibited the BK-induced $[Ca^{2+}]_i$ elevation and force in RSV in a non-competitive manner. B_2-receptor mRNA was detected by RT-PCR, as shown in Figure 3c. Since it is well known that the BK receptor of the vascular endothelial cells is the B_2 subtype (Schini *et al*., 1990; Hock *et al*., 1991; Hecker *et al*., 1992; Mombouli & Vanhoutte, 1992; Drummond & Cocks, 1995), we paid special attention to remove endothelial cells, as described in the Tissue preparation section, in the present study. Ma *et al*. (1994) have already obtained the expression of B_2-receptor mRNA in the mouse aorta and vein. However, they did not specify whether or not they removed endothelial cells. We provide evidence for the expression of both the functioning B_2-receptors and their mRNA in vascular smooth muscle cells.

In summary, the present study demonstrated that: BK elicits vasoconstriction of RSV by (1) increasing Ca^{2+} sensitivity of the contractile apparatus and (2) elevating $[Ca^{2+}]_i$ as a result of Ca^{2+} influx from the extracellular space and Ca^{2+} release from intracellular thapsigargin-sensitive storage site. The increase in the Ca^{2+} sensitivity is mediated by G-protein. (3) BK-induced contractions are mediated via B_2-receptors and B_2-receptor mRNA is expressed in RSV smooth muscle cells.

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