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Direct activation of endothelial NO pathway by Ba^{2+} in canine coronary artery

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1 We have reported that Ba^{2+} causes endothelium-dependent relaxation of canine coronary arteries through NO synthesis in Ca^{2+}-free and depolarizing solution. To determine the cellular mechanisms by which the endothelium-dependent relaxation occurs, we used fura-2 fluorometry (F_{350} and F_{390}; excitation wavelengths, 350 and 390 nm, respectively) and estimated the intracellular Ba^{2+} concentration in endothelial and vascular smooth muscle cells.

2 Ba^{2+} (10^{-3} M) increased the fura-2 ratio (F_{350}/F_{390}) recorded from a combined preparation of smooth muscle and endothelium (0.445 ± 0.073, $n=4$) and contracted the arteries in the presence of 80 mM K^+ (0.22 ± 0.06 g, $n=4$).

3 Diltiazem (3×10^{-6} M) blocks Ba^{2+} entry into vascular smooth muscle cells via L-type Ca^{2+} channels. In this condition, Ba^{2+} increased the fura-2 ratio in endothelial cells (0.141 ± 0.014, $n=5$) and relaxed the underlying smooth muscle (0.08 ± 0.01 g, $n=5$) by a mechanism which was sensitive to 10^{-4} M N^G-methyl-L-arginine (L-NMMA).

4 Ba^{2+}-induced relaxation was not attenuated with repeated application and was elicited even after endothelium-dependent relaxations in response to 10^{-6} M bradykinin were abolished due to tachyphylaxis. Neither 10^{-2} M caffeine nor 10^{-6} M thapsigargin had effect upon Ba^{2+}-induced relaxation.

5 To further rule out changes in intracellular Ca^{2+} as a mechanism of Ba^{2+}-induced relaxation, fura-2 fluorescence was measured at the isosbestic wavelengths for Ca^{2+} (360 nm) and Ba^{2+} (370 nm) in endothelium-intact arteries. Ba^{2+} altered F_{360}, but not F_{370}, suggesting little or no contribution of intracellular Ca^{2+} to the phenomenon of Ba^{2+}-induced relaxation.

6 These results suggest that the Ba^{2+}-induced relaxation is due to its direct activation of endothelial NO synthesis without mobilization of intracellular Ca^{2+}.

Keywords: Barium; calcium; nitric oxide; fura-2; endothelium; smooth muscle; coronary artery

Introduction

Divalent metal cations are useful tools in the study of plasmalemmal Ca^{2+} influx pathways. Among them, Ba^{2+} has interesting biochemical properties as it permeates plasmalemmal Ca^{2+} channels but does not refill intracellular stores (Kwan & Putney, 1990). Ba^{2+} can bind to a Ca^{2+}-sensitive dye, like fura-2, in a reversible manner, and so protocols can be designed to determine whether the effect of a certain compound is washed out during the fluorescence measurement. In contrast, some of the transition divalent cations such as Mn^{2+} can also enter cells, and their binding to the Ca^{2+}-sensitive dye quenches the fluorescence (Jacob, 1990). In this respect, Ba^{2+} may be a better tool to examine Ca^{2+} influx pathways physiologically and pharmacologically in different types of living cells.

There have been a number of documents reporting the biological actions of Ba^{2+}. This cation has a lower affinity for the Ca^{2+} binding protein calmodulin because of its smaller ion radius compared to that of Ca^{2+} (Chao *et al*., 1984). Regardless of its lower affinity for calmodulin, Ba^{2+} can replace Ca^{2+} in contracting various vascular smooth muscles under depolarizing conditions (Hansen *et al*., 1984; Ebeigbe & Aloamaka, 1985; Karaki *et al*., 1986; Kreye *et al*., 1986; Satoh *et al*., 1987). In these studies, it has been proposed that Ba^{2+} enters smooth muscle cells through L-type Ca^{2+} channels and activates the contractile elements directly by binding to calmodulin and/or acts indirectly to release Ca^{2+} from intracellular stores. Many vasoactive compounds proposed to regulate smooth muscle tone have opposite actions on endothelium and smooth muscle (see Himmel *et al*., 1993 for review). Ba^{2+} is known to enter cultured endothelial cells by the same route as Ca^{2+} (Schilling *et al*., 1989); therefore we speculate that this cation can produce some relaxing factors and modify the muscle tone in an opposite manner to its well-known action on smooth muscle cells.

Previously, we found that in the presence of diltiazem, Ba^{2+} can replace Ca^{2+} in relaxing depolarized canine and porcine coronary arteries through activation of nitric oxide (NO) synthesis in endothelium (Ohashi *et al*., 1995; Yamazaki *et al*., 1995). Our recent biochemical study suggests that Ba^{2+} can activate NO synthase directly in the absence of Ca^{2+} (Yamazaki *et al*., 1996). Therefore, it is conceivable that Ba^{2+} can decrease smooth muscle tone by endothelial NO production after complete blockade of L-type Ca^{2+} channels in smooth muscle. However, from our previous studies, the possibility that Ca^{2+} released from intracellular stores may be involved in this phenomenon could not be ruled out, since an important role for intracellular Ca^{2+} stores is known in agonist-induced NO release. Endothelial Ca^{2+} increase depends on the release of Ca^{2+} from inositol 1,4,5-triphosphate (IP_3)-sensitive Ca^{2+} stores, followed by a Ca^{2+} influx through a stimulated capacitative Ca^{2+} entry pathway (Schilling *et al*., 1992; Dolor *et al*., 1992). The resultant

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increase in the Ca^{2+} level in the endothelium is a crucial step in the production of NO (Busse & Mülsch, 1990; Forstermann *et al*., 1991).

Simultaneous measurement of intracellular divalent cations in endothelium and the tension in smooth muscle is a practical approach for testing the hypothesis that Ba^{2+} influx into endothelial cells modulates vascular tone without mobilization of intracellular Ca^{2+}. In this study, we have modified our previous published techniques (Sato *et al*., 1998) and simultaneously measured fura-2 fluorescence and tension in the depolarized canine coronary artery. To eliminate the possibility of involvement of intracellular Ca^{2+} stores in the mechanism of Ba^{2+}-induced relaxation, in addition to manipulation of Ca^{2+} stores with bradykinin, caffeine and thapsigargin, we employed an improved method to define solely the increase in Ba^{2+} by comparing the emission light evoked by the different isosbestic excitation wavelengths for Ca^{2+} and Ba^{2+}.

Methods

Chemicals

The drugs used in this study were: $BaCl_2$, cremophor EL (Nacalai Tesque Co. Ltd., Japan), $CaCl_2$, $CdCl_2$, $NiCl_2$, $LaCl_3$, caffeine, papaverine (Wako Pure Chemicals Co. Ltd., Japan), bradykinin, thapsigargin (Sigma, MO, U.S.A.), diltiazem (Tanabe Seiyaku Co. Ltd., Japan), tetrakis(2-pyridylmethyl) ethylene-diamine (TPEN, Molecular Probes Co. Ltd., U.S.A.), fura-2/AM and fura-2 pentapotassium salt (Dojin Chemical Laboratories Co. Ltd., Japan). N^G-Monomethyl-L-arginine acetate (L-NMMA) was synthesized at the Organic Chemistry Research Laboratories (Tanabe Seiyaku Co. Ltd., Japan). Fura-2/AM, bradykinin and thapsigargin were prepared as a stock solution in dimethyl sulfoxide (DMSO). The final concentration of DMSO was less than 0.1% v/v, which itself did not affect the present experiments. The other compounds were directly dissolved in distilled water.

Preparations

Mongrel dogs (12–30 kg) were anesthetized with pentobarbital Na (30 mg kg^{-1}, i.v.) and killed by exsanguination. All animals received proper care in compliance with the *Guiding Principles for the Care and Use of Laboratory Animals*, approved by The Japanese Pharmacological Society. The heart was immediately excised and the circumflex branch of the left coronary artery (outer diameter, 2.0–2.5 mm) was dissected. The artery was cleared of adhering connective tissue and cut into strips (1.5×10 mm) or ring segments (3.5–4.5 mm long) in physiological salt solution (PSS) containing (in mM): NaCl, 136.9; KCl, 5.4; $CaCl_2$, 1.5; $MgCl_2$, 1.0; HEPES, 20; glucose, 5.5 (pH = 7.4) and aerated with 100% O_2. In denuded preparations, the endothelium was mechanically removed by gentle rubbing of the intimal surface with a cotton swab.

Isometric tension measurement in arterial ring preparation

The rings were suspended in organ chambers filled with 100% O_2-aerated PSS at 37°C. Basal tension was adjusted to 1.5 g. Isometric tension was measured with a strain gauge transducer (UR-10GR, UR-20GR; Minebea, Japan) and recorded on a pen recorder (FBR252A, Toa Denpa, Japan). After equilibration for 20–30 min, aliquots of 40 mM KCl were repeatedly added to obtain steady responses from artery rings. The presence of endothelium was checked by the vasodilator action of acetylcholine (10^{-6} M) in arteries precontracted by 2×10^{-6} M prostaglandin $F_{2\alpha}$; the vasodilation was >60% of the prostaglandin $F_{2\alpha}$-induced contraction. Muscle rings were then equilibrated in nominally Ca^{2+}-free, 80 mM K^+-containing PSS (no Ca^{2+}-chelating agent was added) for more than 10 min. Thereafter, 3×10^{-6} M diltiazem was added to the baths to block Ca^{2+} uptake in smooth muscle. Cd^{2+}, Ni^{2+} and La^{3+} were added 20 min after application of Ba^{2+}. In experiments to determine the reproducibility of Ba^{2+}-induced relaxation, Ba^{2+} (10^{-3} M) was repeatedly applied to the rings at an interval of approximately 30 min. In experiments to test the effects of depletion of intracellular Ca^{2+} stores, either bradykinin, caffeine or thapsigargin were used. The Ba^{2+} (10^{-3} M)-induced relaxation was measured before and after repeated treatments with bradykinin or caffeine and a single application of thapsigargin.

Simultaneous measurement of intracellular Ca^{2+} and Ba^{2+} levels and muscle tension

Cytosolic Ca^{2+} and Ba^{2+} were measured with the Ca^{2+}-sensitive dye fura-2 (Grynkiewicz *et al*., 1985). Muscle strips were loaded with 7×10^{-6} M fura-2/AM for 4–5 h in PSS at room temperature. During the loading procedure, a non-cytotoxic detergent, cremophor EL (0.02% v/v), was added to increase the solubility of fura-2/AM. To eliminate the possible quenching effect of endogenous heavy metal ions, 10^{-5} M TPEN was also added. Following the loading period, the strips were rinsed with PSS for 30 min.

The fura-2 loaded muscle strip was incubated with PSS and maintained at 37°C and aerated with 100% O_2. Fluorescence was measured by pinning one end of the strip to a silicon rubber sheet on the bottom of the organ bath (5 ml volume) and mounted on a dual excitation fluorescence spectrophotometer (CAF-110, Japan Spectroscopic, Japan) (Sato *et al*., 1990). The other end of the muscle strip was connected to a strain gauge transducer (SB-1T, Nihon Kohden, Japan) for the measurement of isometric tension with a resting tension of 1 g. The muscle strip was exposed alternatively (128 Hz) to the two excitation wavelengths (350 and 390 nm) and the emission wavelength (500 nm) was collected through a photomultiplier tube. This combination of excitation wavelengths allowed the greatest ratio change during the measurement of Ba^{2+} signals to be obtained. The F_{350}/F_{390} ratio was used as an index of intracellular Ba^{2+}. In order to measure a signal with more fluorescence emitted from endothelial cells than from smooth muscle cells, the endothelial surface was exposed to both excitation wavelengths. In some experiments, the optimal excitation wavelengths for Ca^{2+} (340 and 380 nm) and isosbestic wavelengths for Ca^{2+} (360 nm) and Ba^{2+} (370 nm) were used.

In the present study, there were two types of vessel preparations: endothelium-intact (intact preparation) and endothelium-denuded (denuded preparation) arteries. Muscle strips were equilibrated in nominally Ca^{2+}-free, 80 mM K^+-containing PSS. Thereafter, in some preparations, 3×10^{-6} M diltiazem was added to the baths to block Ca^{2+} uptake in smooth muscle. Ten minutes following the addition of diltiazem, Ca^{2+} (10^{-3} M) or Ba^{2+} (10^{-3} M) was applied to the baths. The NO synthase inhibitor, L-NMMA (10^{-4} M) was applied 5 min prior to the addition of diltiazem. In the other preparations, Ba^{2+} (10^{-3} M) was applied at least 20 min after stabilization of muscle tension in the nominally Ca^{2+}-free, 80 mM K^+-containing PSS.

Characteristics of the fluorescent dye fura-2 bound to Ca^{2+} and Ba^{2+} (Non-biological system)

Ca^{2+}- and/or Ba^{2+}-EGTA buffers at various concentrations were prepared (in mM: NaCl, 25, KCl 125, EGTA, 3; HEPES, 20; 37°C; pH = 7.4) according to the software for calculating concentrations of free divalent cations (Schoenmakers *et al*., 1992). Fura-2 pentapotassium salt (10^{-6} M) was dissolved in the buffer. The excitation spectra for Ca^{2+}- and Ba^{2+}-fura-2 complexes were measured with a spectrophotometer (F2000, Hitachi, Japan) when the emission wavelength was set at 500 nm. Fluorescence (emission, 500 nm) from the Ca^{2+} and Ba^{2+} buffer evoked by optimal (Ca^{2+}, 340 and 380 nm; Ba^{2+}, 350 and 390 nm) and isosbestic wavelengths (Ca^{2+}, 360 nm; Ba^{2+}, 370 nm) was measured with a dual excitation fluorescence spectrophotometer (CAF-110, Japan Spectroscopic, Japan).

Statistics

Results are expressed as means ± s.e.mean. Statistical analysis was conducted by using the software SigmaStat 2.0 (SPSS Science, IL, U.S.A.). Comparisons between two groups were carried out using unpaired Student's or Welch's *t*-test. Analysis of variance (ANOVA) and Bonferroni's multiple *t*-test were used to compare more than 2 groups. One-way ANOVA with repeated measures and *post hoc* Bonferroni's *t*-test was performed to compare the repeated application of Ba^{2+}. An unpaired *t*-test was used for comparison of the second response to bradykinin to the first response. Values of $P<0.05$ were considered significant.

Results

Excitation spectra of Ca^{2+} and Ba^{2+}-fura-2 complexes (Non-biological system)

Excitation spectra (from 300 to 420 nm) for fura-2 was obtained by measuring the intensity of emission light at 500 nm in the presence of various concentrations of Ca^{2+} and Ba^{2+}. Both Ca^{2+} and Ba^{2+} increased the peak of the excitation spectra for the fura-2 salt and shifted it to the low wavelength (Figure 1a and b). Optimal excitation wave-

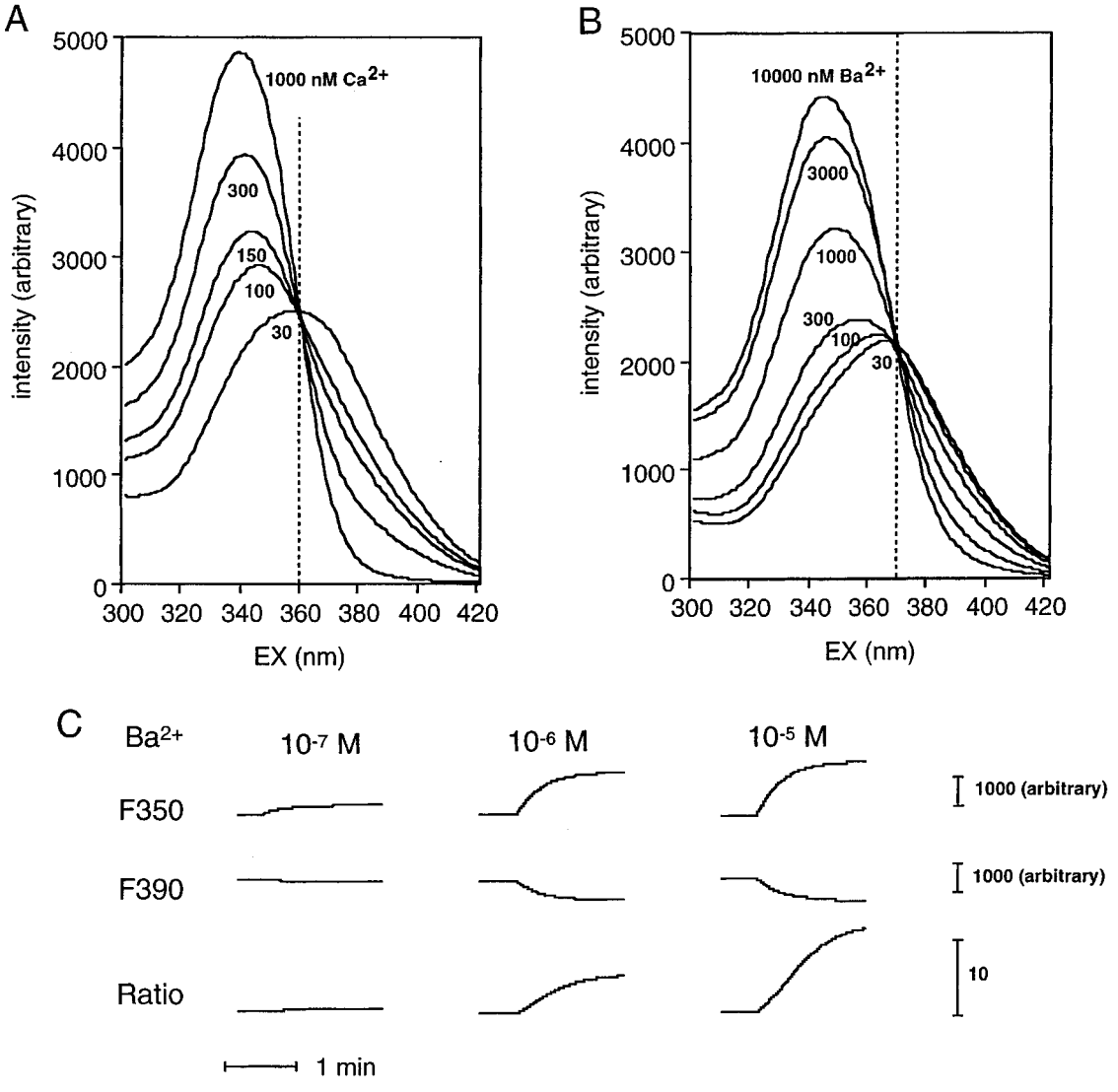

Figure 1 Excitation (EX) spectra of fura-2 at various concentrations (nM) of Ca^{2+} (a) and Ba^{2+} (b). The Ba^{2+}-induced changes in fura-2 fluorescence (F_{350} and F_{390}) and F_{350}/F_{390} ratio (c). Emitted fluorescence was monitored at 500 nm. Dotted line indicates the isosbestic wavelength for each divalent cation. In c, Ba^{2+} was applied to fura-2-containing solution in test tubes.

lengths at which divalent cations caused notable changes in intensity were 350 and 390 nm in the presence of Ba^{2+}, and 340 and 380 nm in the presence of Ca^{2+}. Isosbestic wavelengths for Ca^{2+} and Ba^{2+} were 360 and 370 nm, respectively.

Intensity of emission lights evoked by 350 and 390 nm excitation (F_{350} and F_{390}) was measured in different concentrations of Ba^{2+} with a dual excitation fluorescence spectrophotometer. Changes in intensity and the calculated ratio (F_{350}/F_{390}) are shown in Figure 1c. Addition to Ba^{2+} (10^{-7}, 10^{-6} and 10^{-5} M) to fura-2 salt (10^{-6} M)-containing solution increased F_{350}, decreased F_{390} and increased the F_{350}/F_{390} ratio in a concentration-dependent manner. These basic findings suggest that a combination of excitation light at 350 and 390 nm may be optimal for measurement of Ba^{2+} level in living cells as well as in the non-biological system.

Ba^{2+}-induced changes in fluorescence and muscle tension in canine coronary arteries

The F_{350}/F_{390} ratio derived from smooth muscle with or without endothelium, and the muscle tension were measured simultaneously in depolarized canine coronary arteries.

Absence of a Ca^{2+} channel blocker Firstly, the effect of the addition of Ba^{2+} was studied in the absence of diltiazem (Figure 2). Ba^{2+} caused an increase in the F_{350}/F_{390} ratio and muscle tension in the intact preparation in the 80 mM K^+-containing PSS. Removal of endothelium caused the contraction to become slightly stronger 15 min after the addition of Ba^{2+}, although this change was not statistically significant (0.22 ± 0.06 g in the intact; 0.25 ± 0.08 g in the denuded preparation; unpaired *t*-test, $P > 0.05$; $n = 4$). The increase in F_{350}/F_{390} ratio 15 min after the addition of Ba^{2+} (0.368 ± 0.103; $n = 4$) appeared to be less in the denuded preparation than in the intact preparation (0.445 ± 0.073; $n = 4$), although these values were not significantly different (unpaired *t*-test, $P > 0.05$).

Presence of a Ca^{2+} channel blocker Diltiazem (3×10^{-6} M) itself had little effect on the basal tension (-0.01 ± 0.02 g, $n = 5$). In the presence of 3×10^{-6} M diltiazem and 80 mM K^+-containing PSS (Figure 3), 10^{-3} M Ba^{2+} increased F_{350} and decreased F_{390}, thus increasing the F_{350}/F_{390} ratio (0.141 ± 0.014, $n = 5$), and relaxed the smooth muscle (0.08 ± 0.01 g, $n = 5$) in the intact preparation. Figure 4a and b show the mean values of time courses for muscle tension and the F_{350}/F_{390} ratio. Ba^{2+}-induced relaxation was inhibited in denuded preparations and intact preparations pretreated with the NO synthase inhibitor, L-NMMA (10^{-4} M). At 15 min following addition of Ba^{2+}, the decrease in muscle tension in the denuded and L-NMMA-treated preparations were significantly less than in the intact preparation (ANOVA, $P < 0.01$; $n = 5$). Fifteen min after the addition of Ba^{2+}, the increase in F_{350}/F_{390} ratio was significantly inhibited in the denuded preparation when compared to the intact preparation (ANOVA, $P < 0.01$; $n = 5$). In contrast, F_{350}/F_{390} ratio was not altered in L-NMMA-treated preparations (ANOVA, $P > 0.05$; $n = 5$). These results suggest that an increase in divalent cation, supposedly Ba^{2+}, in endothelial cells precedes NO production and the resultant relaxation of coronary arteries.

Inhibitory effects of inorganic Ca^{2+} influx inhibitors on the Ba^{2+}-induced relaxation

To ascertain that Ba^{2+}-influx into endothelium triggered relaxation, the effects of inorganic cations on the Ba^{2+}-induced relaxation in endothelium-intact canine coronary arteries were examined. Figure 5a shows the inhibition of Ba^{2+}-induced relaxation in the depolarized coronary arterial rings by Cd^{2+} in a concentration-dependent manner. Figure 5b summarizes the inhibitory actions of these cations. Ni^{2+} (10^{-3} M) and La^{2+} (10^{-3} M) also each significantly inhibited the relaxation (ANOVA, $P < 0.01$; $n = 4-6$), but potency for the inhibition of these cations appeared to be less than that of Cd^{2+}.

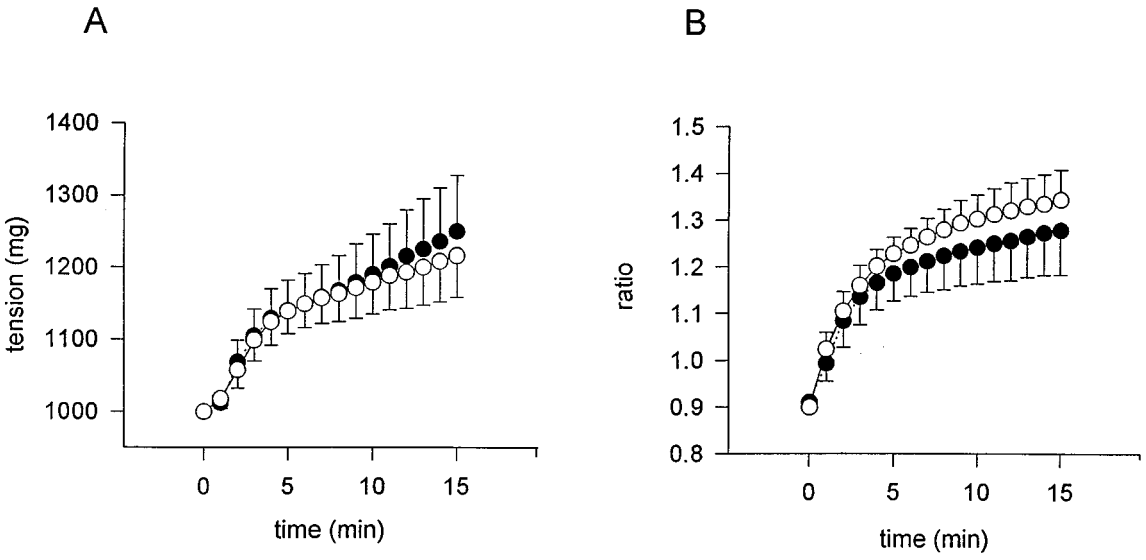

Figure 2 Effects of 10^{-3} M Ba^{2+} on tension (a) and ratio (F_{350}/F_{390}, b) in the absence of diltiazem in canine depolarized (80 mM KCl) arteries loaded with fura-2. The arteries were preincubated in nominally Ca^{2+}-free PSS. (○) intact; (●) denuded. The resting tension was set at 1.0 g before adding Ba^{2+} (a). The baseline values of the F_{350}/F_{390} ratio were 0.899 ± 0.012 and 0.910 ± 0.019 in the intact and denuded preparations, respectively (b). Each point represents the mean of six experiments with s.e.means. No significant difference was found at any period from the controls (unpaired *t*-test, $P > 0.05$).

Effects of repeated application of Ba^{2+} and effects of bradykinin, caffeine and thapsigargin

Previously we have demonstrated that Ca^{2+} causes relaxation and a concomitant increase in intracellular Ca^{2+} level in endothelium of the canine coronary strips (Sato *et al*., 1998). Therefore, Ca^{2+} mobilization could be involved in the effects of Ba^{2+} and cause an indirect action on smooth muscle tension. Two approaches were used to test this possibility in endothelium-intact canine coronary arteries. The first involved an examination of the amplitude of the relaxation evoked by repeated application of Ba^{2+} in the absence of external Ca^{2+} and an examination of the effects of compounds known to affect intracellular Ca^{2+} stores, on the Ba^{2+}-induced relaxation. Repeated application (up to five times) did not cause an apparent decrease in the amplitude of the Ba^{2+} (10^{-3} M) response (Figure 6a). There was no statistical significance among amplitudes in these trials (ANOVA, $P>0.05$). Bradykinin (10^{-6} M) was applied twice to the coronary arteries, and the second response to bradykinin was completely abolished (paired *t*-test, $P<0.01$; Figure 6b inset). However, Ba^{2+}-induced relaxation was not affected by the repeated challenge of bradykinin (Figure 6b). Caffeine (10^{-2} M, 20 min × two times) or thapsigargin (10^{-6} M, 20 min), which is known to cause Ca^{2+} release from different types of Ca^{2+} stores, did not alter Ba^{2+}-induced relaxation (unpaired *t*-test, $P>0.05$; Figure 6c and d).

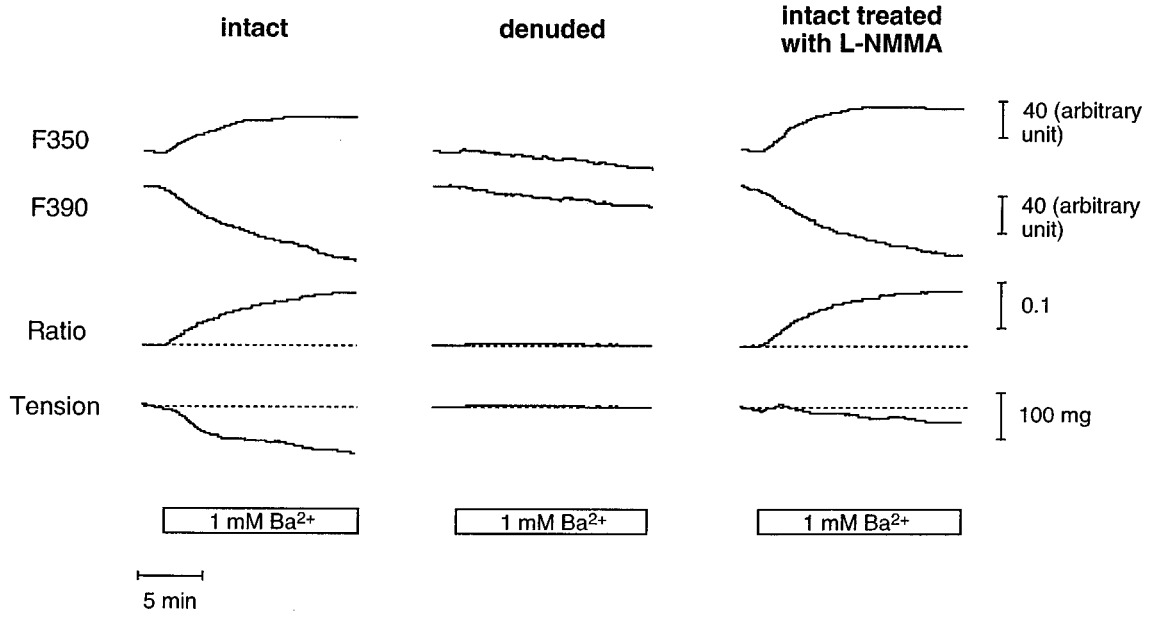

Figure 3 Representative recordings of changes in fluorescence (F_{350} and F_{390} at 500 nm emission), the ratio (F_{350}/F_{390}) and the tension induced by 10^{-3} M Ba^{2+} in depolarized (80 mM KCl) canine coronary arteries. Arteries were preincubated in nominally Ca^{2+}-free solution. Resting tension was set at 1 g and basal values of the F_{350}/F_{390} ratio were 0.86, 0.84 and 0.86 in intact, denuded and L-NMMA-treated arteries, respectively.

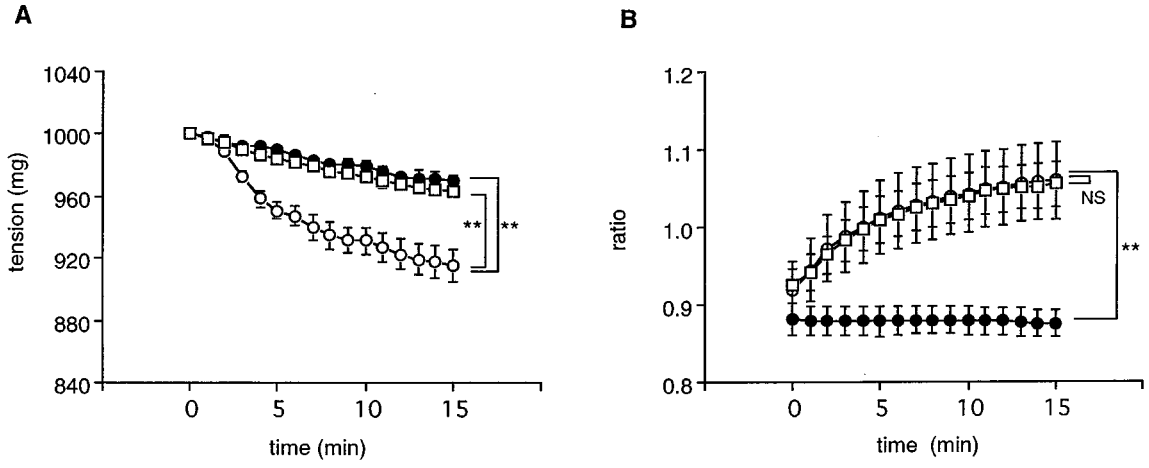

Figure 4 Effects of 10^{-3} M Ba^{2+} on tension and F_{350}/F_{390} ratio in depolarized (80 mM KCl) canine coronary arteries. Ba^{2+} was applied 10 min after addition of 3×10^{-6} M diltiazem. Arteries were preincubated in nominally Ca^{2+}-free solution, and resting tension was set at 1 g. (a) Time course of change in tension; (b) Time course of change in the F_{350}/F_{390} ratio. (○) intact, (●) denuded; (□) intact treated with 10^{-4} M L-NMMA. Each point represents the mean of five experiments with s.e.means. $^{**}P<0.01$ (ANOVA); the tension or F_{350}/F_{390} at 15 min was significantly different from that in the intact arteries. NS; no statistical difference.

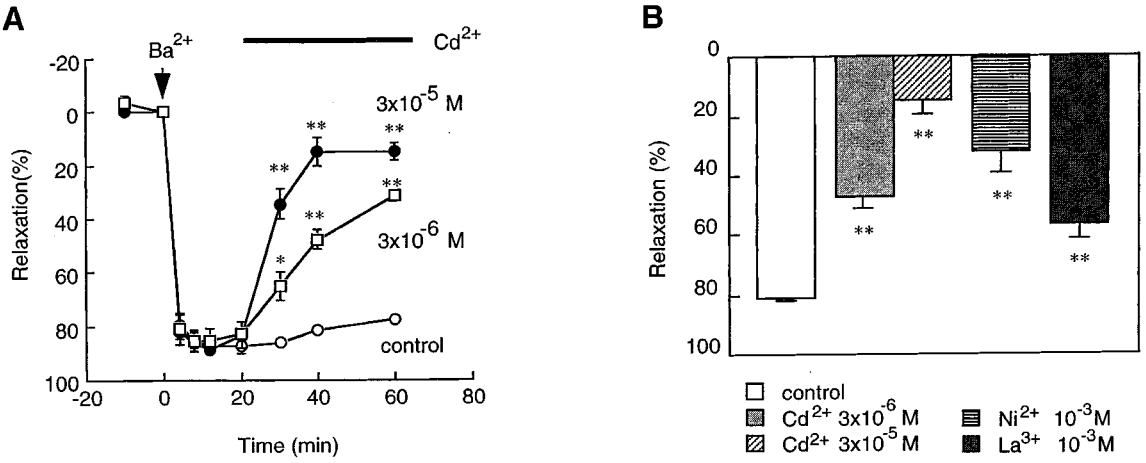

Figure 5 Effects of inorganic Ca^{2+} influx inhibitors on Ba^{2+}-induced relaxation of canine depolarized coronary artery rings. Tissues were exposed to diltiazem (3×10^{-6} M) for 10 min before adding Ba^{2+} (10^{-3} M). Arteries were preincubated in nominally Ca^{2+}-free solution. Resting tension was set at 1.5 g. Cd^{2+}, Ni^{2+} or La^{3+} were each added when the Ba^{2+} response was maximum. Average response to Cd^{2+}, Ni^{2+} or La^{3+} is shown in (b). Percent relaxation relative to the maximal relaxation by papaverine (10^{-4} M) is indicated on the ordinate. Each point represents the mean of four to six experiments with s.e.means. $*P<0.05$, $**P<0.01$ (ANOVA); compared with the control.

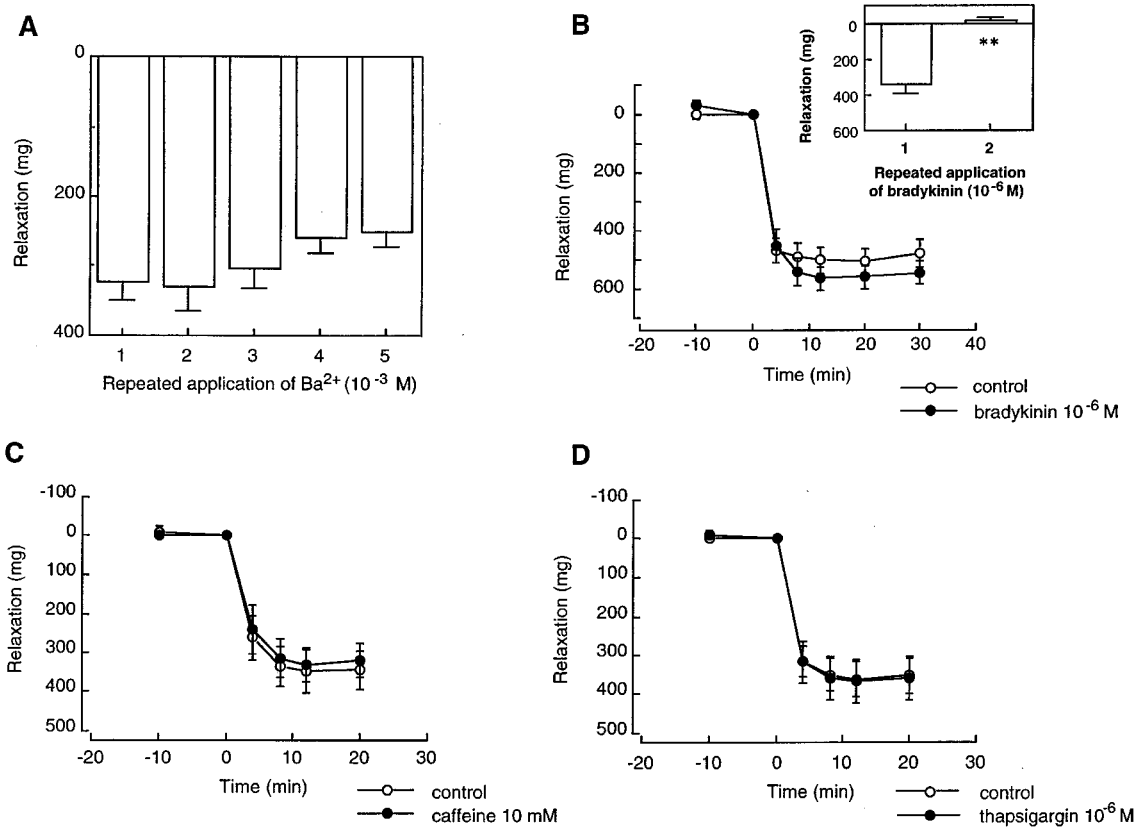

Figure 6 Reproducible relaxation of depolarized canine coronary artery rings in response to Ba^{2+} (a) and effects of treatment with bradykinin (b), caffeine (c) and thapsigargin (d) on the Ba^{2+}-induced relaxation. Tissues were exposed to diltiazem (3×10^{-6} M) for 10 min before adding Ba^{2+} (10^{-3} M). Arteries were preincubated in nominally Ca^{2+}-free solution, and resting tension was set at 1.5 g. Each point represents the mean of four to five experiments with s.e.mean. (a) Ba^{2+} (10^{-3} M) was intermittently applied five times at an interval of approximately 30 min. No significant difference was found from the first response (ANOVA with repeated measures, $P>0.05$). (b–d) Ba^{2+} (10^{-3} M) was added before and after two treatments with bradykinin (b) or caffeine (c), or a single treatment with thapsigargin (d). No significant difference was found at any period from the controls (unpaired t-test, $P<0.05$). Inset figure indicates the dilatatory responses to bradykinin. $**P<0.01$ (paired t-test); compared with the first response.

Ca^{2+}- and Ba^{2+}-induced relaxation and change in fura-2 fluorescence at isosbestic wavelengths

The second approach to examine whether Ca^{2+} mobilization from intracellular stores could be involved in the effects of Ba^{2+} was to measure the fluorescences at the isosbestic wavelengths for Ca^{2+} (360 nm) and Ba^{2+} (370 nm) (see Figure 1c). First of all, we examined fura-2 signals which were evoked at the isosbestic wavelengths in the presence of a combination of Ca^{2+} and Ba^{2+} in the non-biological system in order to define whether it is possible to distinguish the emission light due to Ba^{2+}-fura-2 from that due to Ca^{2+}-fura-2. Figure 7a shows typical traces of fluorescence from Ca^{2+} and Ba^{2+} buffer in the response to excitation light at each isosbestic wavelength. Ca^{2+} (10^{-7} M) did not alter F_{360}, although Ba^{2+} (10^{-7}, 10^{-6} and 10^{-5} M) increased the fluorescence. On the other hand, Ba^{2+} (10^{-7}, 10^{-6} and 10^{-5} M) did not alter F_{370} but Ca^{2+} (10^{-7} M) decreased the fluorescence. Therefore, instead of the narrow range of excitation wavelength, these characteristics of the fura-2 signal can be used to define which divalent cation mostly binds to fura-2. If only Ca^{2+} increases, then F_{370} should change; if only Ba^{2+} increases, then F_{360} should change.

In the biological system, however, endogenous Ca^{2+} contaminates, and the fura-2 fluorescence due to Ba^{2+} is supposed to be considerably affected thereby. To investigate the effect of the coexistence of Ca^{2+} in the Ba^{2+}-containing solution on emission light, fluorescence evoked by excitation light at the isosbestic wavelengths for Ca^{2+} and Ba^{2+} was measured in a non-biological system when the concentration of Ba^{2+} was increased to 10^{-5} M in the presence of 10^{-7} M Ca^{2+} (Figure 7b). Although more than 10^{-7} M Ba^{2+} increased F_{360} in the absence of Ca^{2+} (Figure 7a), even 10^{-5} M Ba^{2+} did not show any obvious change in F_{360} in the presence of Ca^{2+}. Instead, the typical decrease in F_{370} by Ca^{2+} was obtained even in a 100 fold lower concentration than Ba^{2+}. Therefore, where Ba^{2+} causes a Ca^{2+} release from intracellular stores, resulting in an increase of Ca^{2+} concentration at least to the value of 100 fold less than the Ba^{2+} concentration, then the pattern of the fura-2 signals with the mixture of both cations should follow the signal pattern with Ca^{2+} alone. In other words, if F_{370} is not altered during the experiment, then involvement of Ca^{2+} mobilization may be assumed negligible.

The resting tension of the K^+-depolarized canine coronary arteries was set at 1 g in the presence of diltiazem. F_{360} and F_{370} tended to decline at a constant rate of $-0.30\pm0.04\%$ min^{-1} and $-0.25\pm0.03\%$ min^{-1}, respectively (the control values). Both 10^{-3} M Ca^{2+} and Ba^{2+} caused relaxation (Figure 7c) as shown in the previous paper (Yamazaki *et al.*, 1995). Ba^{2+}-induced relaxation appeared to be greater in amplitude than Ca^{2+}-induced relaxation, although there was no statistical significance (at 15 min; unpaired *t*-test, $P>0.05$). A striking difference was the change in fluorescence (Figure 7d); Ca^{2+} kept declining F_{360} (the isosbestic wavelength for Ca^{2+}) at the same constant ($-0.29\pm0.05\%$ min^{-1}, unpaired *t*-test, $P>0.05$ vs control), although it significantly accelerated F_{370} (the isosbestic wavelength for Ba^{2+}) at a declining constant of $-0.50\pm0.05\%$ min^{-1} (unpaired *t*-test, $P<0.01$ vs control). These results were well explained by the characteristic of excitation spectrum for Ca^{2+}-fura 2 (Figure 7a). On the other

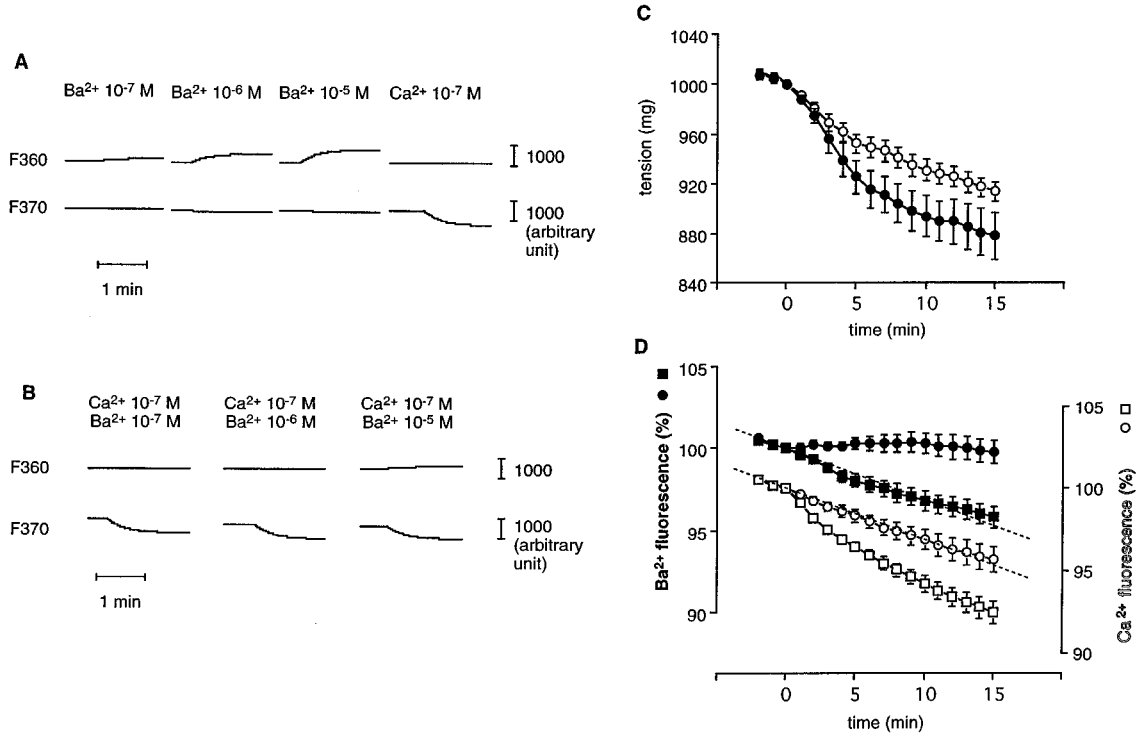

Figure 7 Changes in fura-2 fluorescence (500 nm) evoked at isosbestic wavelengths for Ca^{2+} (360 nm) and Ba^{2+} (370 nm) in the presence of Ca^{2+} or Ba^{2+} (a) and a combination of both divalent cations (b) in test tubes, and simultaneous measurement of tension (c) and the fluorescences (d) in Ca^{2+} and Ba^{2+}-induced relaxation of the depolarized coronary arteries. (a, b) Ca^{2+} and Ba^{2+} were buffered with 3 mM EGTA to make concentrations shown in the figure. Buffer contained 25 mM NaCl, 120 mM KCl, 20 mM HEPES and 1 μM fura-2 salt. (c) Change in tension of the coronary arteries by (○) Ca^{2+} and (●) Ba^{2+}. (d) Change in fura-2 fluorescence evoked by isosbestic wavelengths in the presence of each cation. (○) F_{360}, Ca^{2+}; (□) F_{370}, Ca^{2+}; (●) F_{360}, Ba^{2+}; (■) F_{370}, Ba^{2+}. Each divalent cation was applied to the arteries 10 min after addition of 3×10^{-6} M diltiazem. Arteries were preincubated in nominally Ca^{2+}-free solution. Each value is expressed as percentage of the value at 0 min. Each point represents the mean of 5 experiments with s.e.mean.

hand, Ba^{2+} significantly slowed the decrease in F_{360} at a declining constant of $-0.02\pm0.05\%$ min^{-1} (unpaired *t*-test, $P<0.01$ vs control), although it did not affect the constant for F_{370} ($-0.28\pm0.04\%$ min^{-1}; unpaired *t*-test, $P>0.05$ vs control). If there were any contribution of Ca^{2+} in Ba^{2+}-induced changes in fluorescence, the signal at isosbestic wavelength for Ba^{2+} should have been altered. As shown in the Figure 7b, even though 100-fold less concentration of Ca^{2+} than Ba^{2+} was present in the assay system, the signals entirely follow the characteristics for Ca^{2+}-fura 2; Ca^{2+} is capable of overwhelming the Ba^{2+} binding to fura-2. Nevertheless, the absence of changes in F_{370} observed in the present study indicates that changes in fluorescence or relaxation induced by Ba^{2+} is unlikely to be due to any secondary effect of intracellular Ca^{2+}.

Discussion

An increase in intracellular Ca^{2+} is crucial for the activation of NO synthase in endothelium (Busse & Mülsch, 1990; Forstermann *et al*., 1991). Ca^{2+} entry to endothelium causes relaxation through the NO-cyclic GMP pathway in canine and porcine coronary arteries (Yamazaki *et al*., 1995; Ohashi *et al*., 1995; Sato *et al*., 1998). Since Ba^{2+} enters cultured endothelial cells in response to bradykinin (Schilling *et al*., 1989), it is possible that Ba^{2+} could also move into endothelial cells and relax the intact coronary arteries through production of NO. However, direct evidence has not yet been provided to indicate a correlation between Ba^{2+} influx and Ba^{2+}-induced relaxation. In the present study, evidence to link the movement of Ba^{2+} into the endothelium of intact vessels and the resultant NO-mediated relaxation of smooth muscle of canine coronary arteries has been provided.

In a recent paper, we demonstrated the successful simultaneous measurement of endothelial Ca^{2+} levels and smooth muscle tension (Sato *et al*., 1998). This experiment confirmed that ACh increases the F_{340}/F_{380} ratio (optimal combination of excitation wavelengths for Ca^{2+}) and the corresponding endothelium-mediated relaxation of depolarized smooth muscle strips. The present experimental setup, therefore, allowed us to measure Ca^{2+} influx in the endothelium and the subsequent modulation of the muscle tone. In the present paper, we have modified this methodology for the measurement of Ba^{2+} with two optimal excitation wavelengths (F_{350} and F_{390}) according to the excitation spectrum for Ba^{2+}-fura-2 (Figure 1). Our first aim of the present study was to investigate a possible endothelium-dependent action of Ba^{2+} on the smooth muscle tone, an opposite effect to Ba^{2+}-induced contraction. We found, however, that the removal of the endothelium caused a slight change in smooth muscle contraction and the F_{350}/F_{390} ratio. One difficulty in this may have been due to too strong a contractile response to Ba^{2+} which prevented any clear and definite smooth muscle relaxation mediated by the endothelium from being observed. To circumvent this problem, the Ca^{2+} channel blocker diltiazem was used to block Ca^{2+} and Ba^{2+} entry into the smooth muscle and enable us to unmask the modulation of tension by the endothelium. We can also rule out any effect of diltiazem on endothelial cells since they do not possess voltage-dependent Ca^{2+} channels (Colden-Stanfield *et al*., 1987; Jayakody *et al*., 1987). Moreover, it seems to be advantageous that the resting tone is still preserved under our experimental condition in the presence of diltiazem and nominally Ca^{2+}-free PSS (Kikkawa *et al*., 1989; Yamazaki *et al*., 1994; Yamazaki *et al*., 1995).

In the presence of diltiazem, Ba^{2+} caused a sustained increase in the F_{350}/F_{390} ratio accompanied by a relaxation of smooth muscle in the intact preparation. The increase in the F_{350}/F_{390} ratio and the relaxation were significantly inhibited in the denuded preparation, suggesting that an increase in the ratio and the muscle relaxation are entirely dependent on endothelial cells. Several di- and trivalent cations are known to inhibit the Ca^{2+} influx pathways in endothelial cells. La^{3+} (Schilling *et al*., 1989; 1992) and Ni^{2+} (Dolor *et al*., 1992; Lückhoff & Clapham, 1992) are potent inhibitors of this pathway. Cd^{2+} is reported to inhibit the substance P-induced endothelium-dependent relaxation in dog cerebral arteries treated with indomethacin (Toda & Okamura, 1992). Ni^{2+} and La^{3+} also inhibit Ba^{2+} influx in response to several vasodilators such as bradykinin in endothelial and lacrimal acinar cells (Schilling *et al*., 1989; Kwan & Putney, 1990). In the present study, Cd^{2+}, Ni^{2+} and La^{3+} attenuated the Ba^{2+}-induced relaxation. This strengthens the hypothesis that Ba^{2+} influx to endothelium triggers this relaxation. In the intact preparation, pretreated with the NO synthase inhibitor L-NMMA, Ba^{2+}-induced relaxation was significantly inhibited but the fura-2 signal remained unaffected. These results suggest that the cytosolic divalent cation level, probably that of Ba^{2+}, of endothelial cells increases initially and this leads to the production of NO, which subsequently leads to smooth muscle relaxation.

Although an increase in fura-2 fluorescences and smooth muscle relaxation were successfully measured in the presence of Ba^{2+} in the present study, it was still uncertain whether fura-2 fluorescence was derived solely from Ba^{2+}. It may have been that Ba^{2+} produces NO through an indirect mechanism. For instance, previous studies suggest that Ba^{2+} owes its action to the release of sequestered Ca^{2+} from intracellular stores to explain the phenomenon that Ba^{2+} substitutes for Ca^{2+} in smooth muscle preparations (Ebeigbe & Aloamaka, 1985; Satoh *et al*., 1987). To examine this possibility, two types of experimental protocols were used. The first approach involved an examination of the amplitude of relaxation repeatedly evoked by Ba^{2+}. If intracellular Ca^{2+} stores in endothelium are involved in the mechanism of Ba^{2+}-induced relaxation, then the relaxation should be attenuated by its repetitive application in the absence of external Ca^{2+}. The amplitude of Ba^{2+}-induced relaxation, however, remained almost unchanged in this procedure. More than one type of intracellular Ca^{2+} store is present in endothelial cells. IP_3 that couples with agonist stimulation, such as bradykinin, reportedly releases part of the Ca^{2+} stored in the endoplasmic reticulum (ER) pool (for review, see Himmel *et al*., 1993). Thapsigargin, an inhibitor of the endothelial ER Ca^{2+}-ATPase, which depletes the IP_3-induced Ca^{2+} release store, abolishes agonist-induced NO release from bovine aortic endothelial cells and intact preparation of rabbit aorta and femoral artery (MacArthur *et al*., 1993; Amerini *et al*., 1996). Caffeine can stimulate NO production by the release of Ca^{2+} through a ryanodine-sensitive channel from a Ca^{2+}-induced Ca^{2+} release store in rat aortic endothelium (Hatano *et al*., 1995). In the present study, pretreatment with bradykinin, caffeine or thapsigargin exerted no effect on the Ba^{2+}-induced relaxation. Therefore, IP_3-induced and Ca^{2+}-induced Ca^{2+} stores are not likely to play a significant role in the Ba^{2+}-induced relaxation of canine coronary artery.

In these experiments, however, we could not completely exclude the possibility that a small amount of intracellular Ca^{2+} was still available or that the endothelium-dependent action of Ba^{2+} was obscured by the modulation of Ca^{2+} stores in smooth muscles by bradykinin, caffeine or thapsigargin.

Therefore, we focused on a specific fluorimetric technique to distinguish the Ba^{2+}-induced action from the other actions; that is, by measuring the fluorescence evoked by either the isosbestic wavelength for Ca^{2+} or Ba^{2+}. As shown in the excitation spectrum (Figure 1), F_{360} was not altered by different concentrations of Ca^{2+}, neither was F_{370} by Ba^{2+}. The most important aspect for this measurement is the difference in affinity of these cations for fura-2 (Schilling *et al*., 1989). In fact, our preliminary experiment indicated that F_{360} and F_{370} exhibited a similar change in the combined presence of Ba^{2+} and Ca^{2+} to that seen in the presence of Ca^{2+} alone even though the Ba^{2+} concentration was 100 fold higher than Ca^{2+} (Figure 7b). Therefore, if F_{370} is not altered during the experiment, Ca^{2+} mobilization is considered to be negligible. Indeed, Ba^{2+} was found to change F_{360}, but not F_{370}, in the present study. These results strongly argue for the view that Ba^{2+}-induced relaxation is due to a direct increase in the Ba^{2+} level in the endothelium, and not due to an indirect action secondary to a change in the intracellular Ca^{2+} level.

In our previous paper where the activity of rat cerebellum NO synthase was measured, the effective concentration range for Ba^{2+} was estimated to be 250 times higher than that for Ca^{2+} and the maximal activation by Ba^{2+} was 50–70% of that by Ca^{2+} (Yamazaki *et al*., 1996). This may have been due to the different affinities of these cations for calmodulin. Previous studies have shown that Ba^{2+}, which has a lower affinity for calmodulin, was less effective than Ca^{2+} in eliciting phosphodiesterase activity (Chao *et al*., 1984) and phosphorylation of myosin light chain kinase (Satoh *et al*., 1987). These characteristics of calmodulin for cation selectivity could be similar in endothelial cells. In the present study, although we were not able to estimate the exact concentration of Ba^{2+}, this cation is likely to accumulate in endothelial cells (Schilling *et al*., 1989) as well as in the other types of cells (Graf *et al*., 1982; Gill & Chueh, 1985; Rasgaro-Flores *et al*., 1987; Kwan & Putney, 1990) at levels sufficient to activate NO synthase since Ba^{2+} is not taken up into intracellular stores or readily removed from the cells.

In conclusion, evidence for the movement of Ba^{2+} into the endothelium of intact vessels and the resultant NO-mediated relaxation of smooth muscle of canine coronary arteries have been provided, by improving a fluorimetric method to define solely the increase in Ba^{2+} with a Ca^{2+}-fluorescent dye fura-2. It is likely that Ba^{2+}, like many vasoactive substances, possesses two opposite actions on endothelium and smooth muscle, and mimics the signaling process from influx of Ca^{2+} in endothelium to the regulation of muscle tone through production of NO.

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