

Barium and Strontium Can Substitute for Calcium in Stimulating Nitric Oxide Production in the Endothelium of Canine Coronary Arteries

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ABSTRACT—We investigated whether Ba^{2+} and Sr^{2+} can substitute for Ca^{2+} in stimulating the nitric oxide (NO) production and cause relaxation in vascular smooth muscle. Ba^{2+} and Sr^{2+} , like Ca^{2+} , relaxed K^{+} -depolarized canine coronary arteries in the presence of diltiazem. The Ba^{2+} - and Sr^{2+} -induced relaxation was endothelium-dependent and was largely inhibited by N^G -monomethyl-L-arginine (L-NMMA) and N^G -nitro-L-arginine (L-NNA), but not by indomethacin. These cations increased cyclic GMP levels in the coronary artery to a similar extent, and the increment was completely abolished by L-NMMA. The relaxation induced by each cation was attenuated in the presence of a combination of propranolol, phentolamine and atropine, and L-NNA markedly inhibited any remaining relaxation. This indicates that these cations produce endothelium-dependent relaxation through NO production as well as the relaxation mediated by neurotransmitters. The present study suggests that Ba^{2+} and Sr^{2+} can substitute for Ca^{2+} in the activation of the NO synthase pathway in the endothelium of canine coronary arteries.

Keywords: Barium, Strontium, Nitric oxide, Endothelium, Coronary artery

Nitric oxide (NO) synthases have been shown to catalyze the conversion of L-arginine to NO radicals and L-citrulline in the endothelium (1–3). An increase in intracellular Ca^{2+} is crucial for the activation of NO synthase in endothelial cells in the presence of calmodulin (4, 5). Ba^{2+} and Sr^{2+} have been shown to enter into cultured endothelial cells in response to bradykinin (6). However, no functional role of divalent cations in vascular endothelial cells has as yet been established in contrast to the situation in smooth muscle cells. Ca^{2+} and Sr^{2+} have been shown to cause an endothelium-dependent relaxation of canine coronary arteries contracted by prostaglandin (PG) $\text{F}_{2\alpha}$ in the absence of Mg^{2+} , but Ba^{2+} did not cause such a relaxation (7). On the other hand, Ba^{2+} and Sr^{2+} have been shown to substitute for Ca^{2+} in the contraction of the rat tail artery (8). To our knowledge, no information has been published regarding any relaxant effect of Ba^{2+} produced through endothelial function.

Our previous study has shown that Ca^{2+} produces an endothelium-dependent relaxation of K^{+} -depolarized canine coronary arteries in the presence of Ca antagonists (9, 10). These antagonists were useful in distinguishing the contractile effect of Ca^{2+} on vascular smooth muscle from its relaxant effect through endothelial function. This is because Ca antagonists block L-type Ca^{2+} channels in

smooth muscle, but do not affect the influx of Ca^{2+} into endothelial cells (11). Under these conditions, an increase in Ca^{2+} in the endothelium should have induced production of NO from L-arginine and caused relaxation of the coronary arteries via an increase in cGMP levels. The present study aims to determine whether Ba^{2+} and Sr^{2+} can substitute for Ca^{2+} in producing relaxation through activation of the NO synthase pathway in endothelial cells.

In this study, we measured the tension of and cyclic GMP levels in canine coronary arteries. We demonstrated for the first time that Ba^{2+} caused strong relaxation of K^{+} -depolarized coronary arteries through an NO-dependent mechanism in endothelial cells.

MATERIALS AND METHODS

Chemicals

Drugs used were: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and papaverine HCl (Wako Pure Chemicals Co., Ltd., Osaka); $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ (Kanto Chemical Co., Ltd., Tokyo); $\text{PGF}_{2\alpha}$ Tris salt, N^G -nitro-L-arginine (L-NNA) (Sigma, St. Louis, MO, USA); acetylcholine Cl (Ovisot; Daiichi Pharmaceutical Co., Ltd., Tokyo); diltiazem HCl and atropine sulfate (Tanabe Seiyaku Co., Ltd., Osaka); $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, L-argi-

nine HCl, sodium nitroprusside and EGTA (Nacalai Tesque Co., Ltd., Kyoto); phentolamine mesylate (Regitin; Ciba-Geigy Japan, Ltd., Takarazuka); *dl*-propranolol HCl (Sumitomo Chem. Co., Osaka). *N*^G-Monomethyl-L-arginine acetate (L-NMMA) was synthesized at the Organic Chemistry Research Laboratories (Tanabe Seiyaku Co., Ltd.). A stock solution of L-NNA (3×10^{-2} M) was produced by dissolution in 0.1 N HCl solution. The stock solution of PGF_{2α} Tris salt (10^{-4} M) was produced by dissolution in ethanol. Other compounds were dissolved in distilled water.

Preparations

Mongrel dogs weighing 8 to 18 kg were anesthetized with pentobarbital Na (30 mg/kg, i.v.) and exsanguinated. The heart was immediately excised, and the circumflex branch of the left coronary artery (o.d.: 2.0–2.5 mm) was dissected. The artery was freed from the surrounding connective tissue, and cut into ring segments, 3.5–4.5 mm long. Care was taken not to damage the intimal surface. When required, the endothelium was removed mechanically by gentle rubbing of the intimal surface with a cotton swab. The rings were suspended in organ chambers filled with physiological salt solution (PSS) maintained at 37°C and aerated with 95% O₂ – 5% CO₂. Basal tension was adjusted to 1500 mg. The components of the PSS were: 147.2 mM NaCl, 5.4 mM KCl, 2.2 mM CaCl₂, 1.0 mM MgCl₂, 14.5 mM NaHCO₃ and 5.4 mM dextrose (pH 7.3–7.4). Isometric tension was measured with a strain gauge transducer (UL-10GR, UL-20GR; Minebea, Nagano) and recorded on a pen recorder (FBR252A; Toa Denpa, Tokyo).

Isometric tension measurement

After equilibration, aliquots of 40 mM KCl were repeatedly added to obtain steady responses from the artery rings. The presence of endothelium was determined by the vasodilative action of acetylcholine (10^{-6} M) in arteries precontracted by 2×10^{-6} M PGF_{2α}. The preparation was then repeatedly washed with Ca²⁺-free, K⁺-depolarizing PSS, which consisted of: 72.6 mM NaCl, 80.0 mM KCl, 1.0 mM MgCl₂, 14.5 mM NaHCO₃ and 5.4 mM dextrose (pH 7.3–7.4). As no Ca²⁺-chelating agent was included, "Ca²⁺-free" means nominally Ca²⁺-free. All experiments were performed using Ca²⁺-free, K⁺-depolarizing PSS.

Diltiazem (3×10^{-6} M) was added to block L-type Ca²⁺ channels in smooth muscle. CaCl₂, BaCl₂ or SrCl₂ was added 10 min after the application of diltiazem. L-NMMA was added 20 min after the addition of each cation. L-NNA and indomethacin were added 30 min and 15 min before the addition of each cation, respectively. Propranolol, phentolamine and atropine were added 15

min prior to the addition of each cation. At the end of the experiment, papaverine (10^{-4} M) was added to obtain maximum relaxation. Relaxation due to the cations was expressed as a percentage of the papaverine-induced relaxation. In experiments with endothelium-intact and endothelium-denuded arteries, the decrease in absolute tension was compared between the two groups.

Cyclic nucleotide measurement

Coronary artery ring segments were repeatedly washed with Ca²⁺-free, K⁺-depolarizing PSS (37°C, aerated with 95% O₂ – 5% CO₂). Ten minutes after the addition of diltiazem (3×10^{-6} M), the segments were incubated with each divalent cation for 5 min. L-NMMA was added 5 min before the addition of each cation. Immediately after incubation, each ring was frozen in liquid nitrogen and stored at –70°C. The frozen ring was homogenized with a glass homogenizer in 1.5 ml ice-cold trichloroacetic acid (6%), before centrifugation at $2,000 \times g$ for 10 min at 4°C. The supernatant was collected and extracted with 4 volumes of water-saturated ether before being lyophilized. Each sample was resuspended with 100 μl 50 mM sodium acetate buffer (pH 6.2). Cyclic AMP and cyclic GMP levels were determined radioimmunochemically by a commercially available kit (Yamasa Shoyu Co., Choshi). The protein content was measured according to the method of Lowry et al. (12) using bovine serum albumin (Wako Pure Chemicals Co., Ltd.) as a standard. The cyclic nucleotide content was expressed as fmoles or pmoles per mg protein.

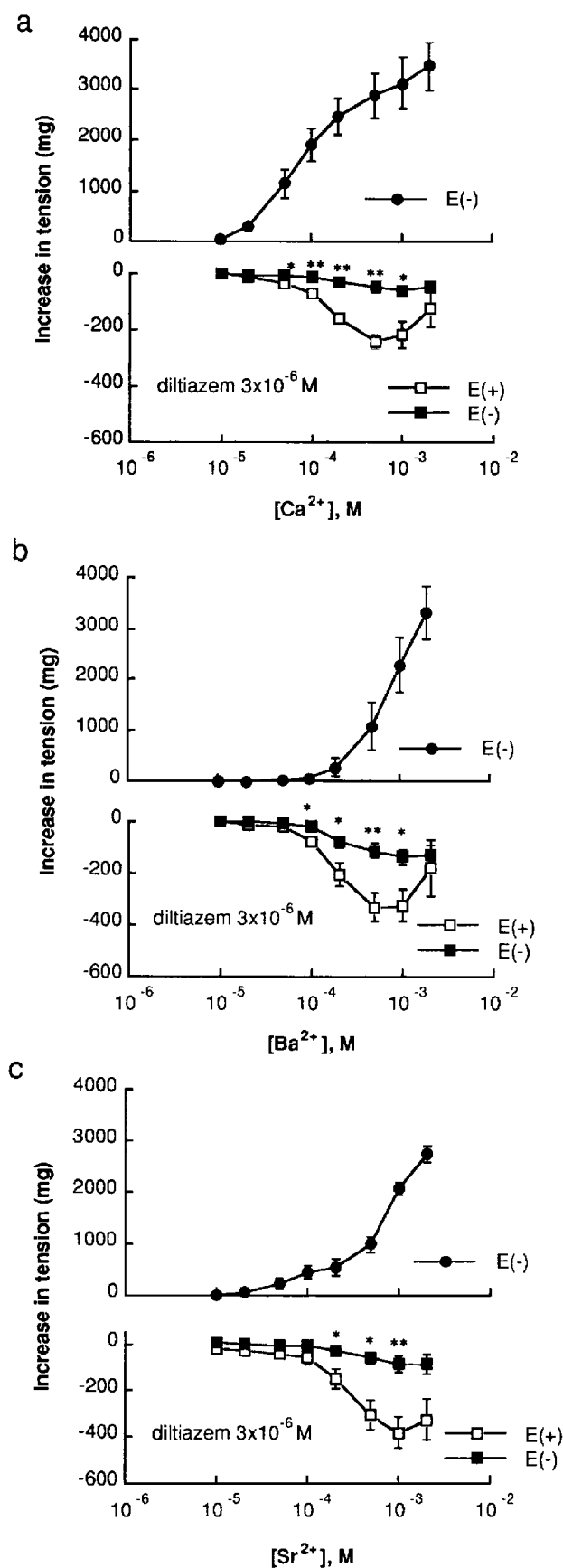
Statistics

Results are expressed as means ± S.E. Statistical comparisons between groups were carried out using Student's *t*-test or Welch's *t*-test. Analysis of variance and Bonferroni's multiple *t*-test were used to compare a control group with more than two other treated groups. Differences at *P* < 0.05 were considered statistically significant.

RESULTS

Basal tone in nominally Ca²⁺-free PSS and divalent cations-induced contraction in canine depolarized coronary artery

The basal tension of the canine coronary artery was adjusted to approximately 1500 mg in the nominally Ca²⁺-free, 80 mM K⁺-depolarizing PSS. Sodium nitroprusside (10^{-6} M), *l*-noradrenaline (10^{-6} M) and papaverine (10^{-4} M) reduced the basal tension by 532.5 ± 55.9 (n=4), 318.3 ± 37.6 (n=6) and 420 ± 52.8 mg (n=7), respectively. These results indicate that the canine coronary artery has active tone in Ca²⁺-free, K⁺-depolarizing PSS. EGTA (2×10^{-3} M) decreased the tension by only



111.4 ± 26.9 mg ($n=7$). An L-type Ca^{2+} channel blocker, diltiazem (3×10^{-6} M), did not significantly decrease the basal tension (5.0 ± 19.6 mg, $n=8$).

Under the above conditions, we compared the contractile response of the depolarized artery to Ba^{2+} and Sr^{2+} with that to Ca^{2+} , in the absence of diltiazem. These cations caused contraction of endothelium-denuded rings in 80 mM K^{+} -depolarizing PSS (Fig. 1). Maximal contraction did not differ in response to Ca^{2+} , Ba^{2+} or Sr^{2+} . However, pEC_{50} values ($n=4$) for Ca^{2+} , Ba^{2+} and Sr^{2+} were different, being 4.0 ± 0.1 , 3.2 ± 0.1 and 3.3 ± 0.1 , respectively. The pEC_{50} value for Ba^{2+} and Sr^{2+} was significantly smaller than that for Ca^{2+} ($P < 0.01$).

Divalent cations-induced relaxation in canine coronary arteries

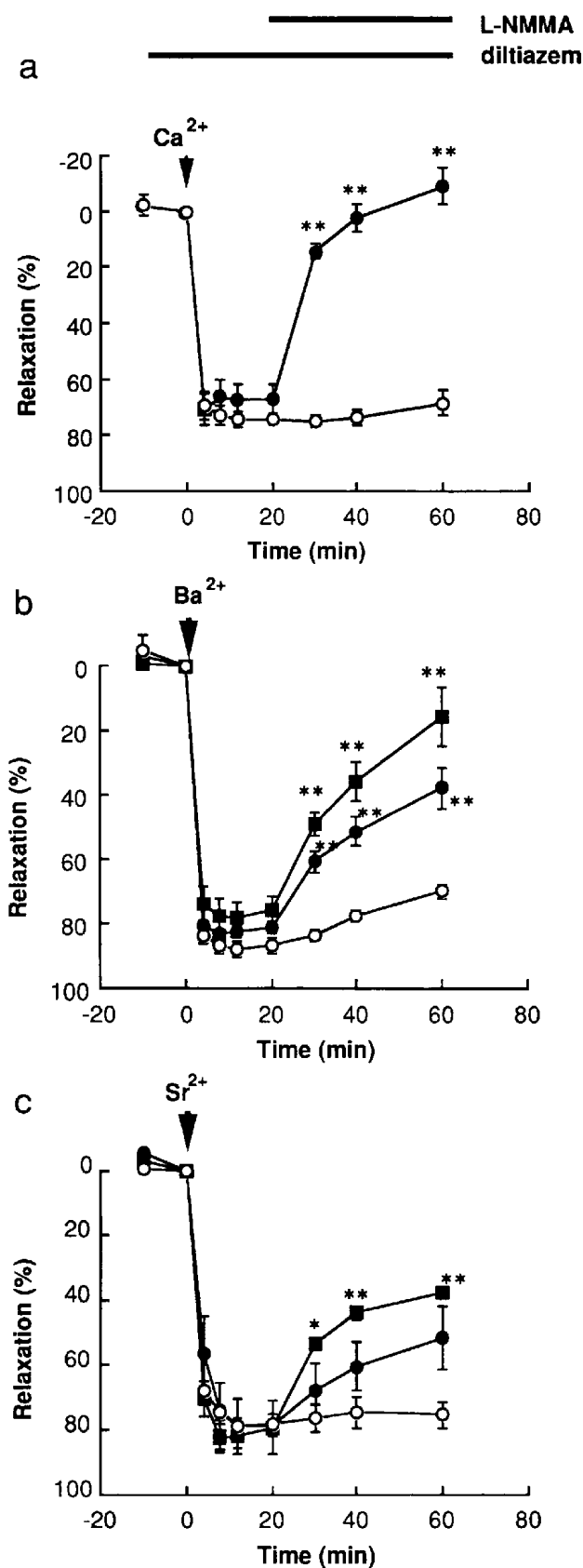
Ba^{2+} and Sr^{2+} , like Ca^{2+} , caused a significant concentration-dependent relaxation of the endothelium-intact rings in K^{+} -depolarizing PSS in the presence of diltiazem (3×10^{-6} M) (Fig. 1). Ca^{2+} -, Ba^{2+} - and Sr^{2+} -induced maximal relaxation was obtained at a concentration of 5×10^{-4} M, 5×10^{-4} M and 10^{-3} M, respectively. Relaxation was attenuated at higher concentrations of each cation. The Ca^{2+} -, Ba^{2+} - and Sr^{2+} -induced relaxation was significantly inhibited in the endothelium-denuded rings as compared with the intact rings in high concentration K^{+} -depolarizing PSS in the presence of diltiazem (Fig. 1).

Effects of L-NMMA, L-NNA and indomethacin

Ca^{2+} (10^{-3} M)-induced relaxation was long-lasting in endothelium-intact arteries (Fig. 2a). Maximal relaxation was 70–80% of that induced by papaverine (10^{-4} M). This relaxation gradually decreased during exposure to Ca^{2+} . Addition of L-NMMA (10^{-4} M) to the coronary artery rings during Ca^{2+} (10^{-3} M)-induced relaxation caused a rapid recovery from relaxation, and sometimes the tone increased above the level of tension before addition of Ca^{2+} .

Ba^{2+} (10^{-3} M)-induced relaxation diminished gradually in endothelium-intact arteries, although more than 75% of the relaxation remained 60 min after the addition of Ba^{2+} (Fig. 2b). L-NMMA (10^{-4} M and 3×10^{-4} M) caused a significant decrease in the Ba^{2+} -induced relaxation. This relaxation was not overcome after blockade by

Fig. 1. Divalent cation-induced contraction and relaxation of canine coronary artery. The arteries were suspended in K^{+} (80 mM)-depolarizing, Ca^{2+} -free PSS. Ca^{2+} (a), Ba^{2+} (b) or Sr^{2+} (c) was applied cumulatively to endothelium-intact (E(+), clear) and -denuded (E(-), solid) arterial tissue in the presence (squares) or absence (circles) of diltiazem (3×10^{-6} M). Each point represents the mean of 4–7 experiments with S.E. * $P < 0.05$, ** $P < 0.01$, compared with the intact artery in the presence of diltiazem.



higher concentrations of L-NMMA.

Sr²⁺ (10⁻³ M)-induced relaxation was long-lasting, and it was maintained 60 min after the addition of Sr²⁺ (Fig. 2c). L-NMMA (3 × 10⁻⁴ M and 10⁻³ M) caused a decrease in Sr²⁺-induced relaxation. The relaxation remained partially present after blockade by a higher concentration of L-NMMA.

Pretreatment with L-NNA (3 × 10⁻⁵ M) completely inhibited Ca²⁺-induced relaxation (Fig. 3a). L-NNA (3 × 10⁻⁴ M) significantly inhibited Ba²⁺- and Sr²⁺-induced relaxation, although relaxation remained partially present as with post-treatment by L-NMMA (Fig. 3, b and c). Indomethacin (5 × 10⁻⁶ M) by itself increased the tension of the coronary arteries by about 75 mg in Ca²⁺-free, high-K⁺ PSS, but did not affect Ca²⁺-, Ba²⁺- and Sr²⁺-induced relaxation (data not shown).

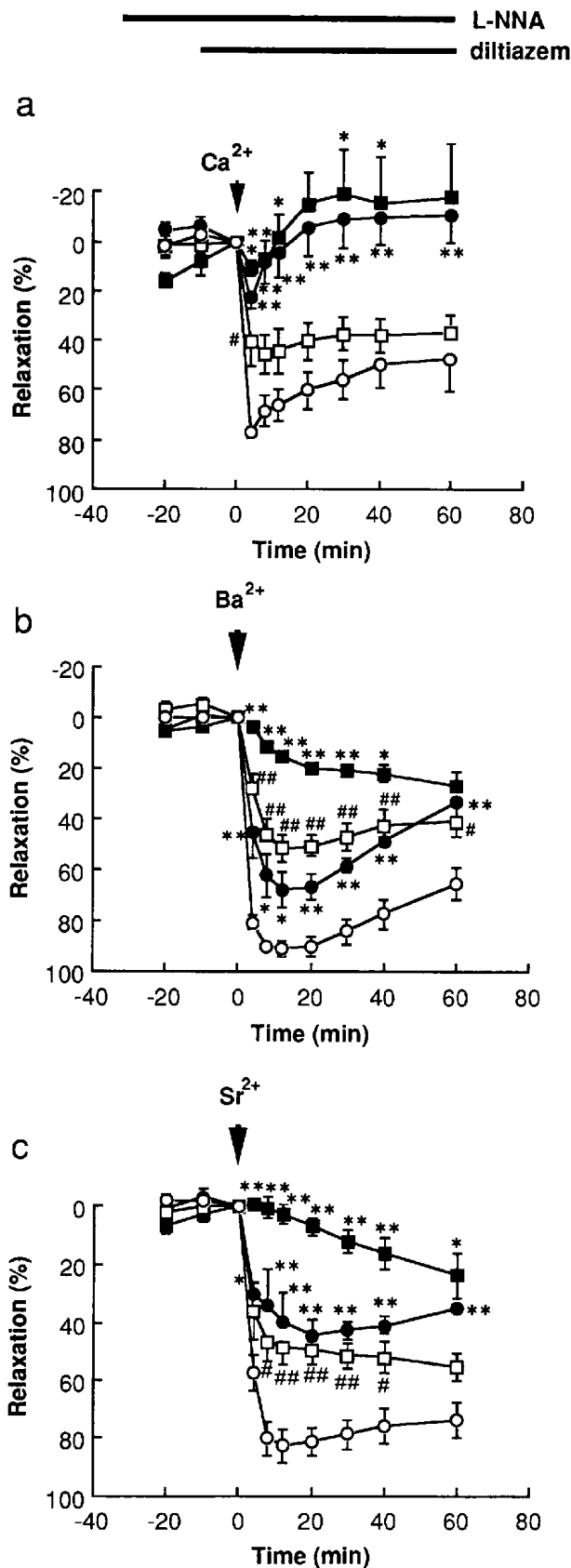
Effects of neurotransmitter antagonists

In the presence of a combination of propranolol (10⁻⁶ M), phentolamine (10⁻⁶ M) and atropine (10⁻⁶ M), Ca²⁺ still caused relaxation of depolarized coronary arteries (Fig. 3a). The degree of relaxation was smaller in the presence than in the absence of these antagonists. The remaining relaxation occurring in the presence of the combination was significantly inhibited by pretreatment with L-NNA. Ba²⁺- and Sr²⁺-induced relaxation was markedly inhibited by the combination of these antagonists throughout the time course of the experiment (Fig. 3, b and c). In the presence of these antagonists, any remaining relaxation was significantly inhibited by pretreatment with L-NNA. However, some degree of Ba²⁺- and Sr²⁺-induced relaxation remained after blockades of both the NO pathway and the neurotransmission.

Divalent cation-induced increase in cyclic GMP levels

Addition of Ca²⁺, Ba²⁺ or Sr²⁺ (10⁻³ M) to the Ca²⁺-free PSS significantly increased the levels of cyclic GMP in K⁺-depolarized coronary arteries (Fig. 4). Pretreatment with L-NMMA (3 × 10⁻⁴ M) significantly inhibited this increase. In contrast to the tension experiments, the Ba²⁺- and Sr²⁺-induced increase in cyclic GMP levels was completely blocked by a modest concentration of L-

Fig. 2. Effects of L-NMMA on Ca²⁺-, Ba²⁺- and Sr²⁺-induced relaxation of canine coronary artery. The arteries were suspended in K⁺ (80 mM)-depolarizing, Ca²⁺-free PSS. Ca²⁺ (a), Ba²⁺ (b) or Sr²⁺ (c) (10⁻³ M) was applied 10 min after addition of diltiazem (3 × 10⁻⁶ M). ○: Control, ●: L-NMMA (10⁻⁴ M), ■: L-NMMA (3 × 10⁻⁴ M). Percentage relaxation relative to the papaverine (10⁻⁴ M)-induced relaxation is shown on the ordinate. Each agent was applied as indicated by the bar. Each point represents the mean of 3–6 experiments with S.E. *P < 0.05, **P < 0.01, compared with the control group.



NMMA. In endothelium-denuded arteries, these cations did not increase cyclic GMP levels beyond the basal level in intact arteries (Ca^{2+} 10^{-3} M, 146 ± 19 fmol/mg; Ba^{2+} 10^{-3} M, 83 ± 9 fmol/mg; Sr^{2+} 10^{-3} M, 82 ± 8 fmol/mg). These cations (10^{-3} M) caused no significant effects on cyclic AMP levels in endothelium-intact arteries (Fig. 4).

DISCUSSION

Substitution of Ba^{2+} and Sr^{2+} for Ca^{2+} in endothelial cells

The main finding of the present study is that Ba^{2+} and Sr^{2+} can substitute for Ca^{2+} in the activation of the NO synthase pathway in the endothelium. We have demonstrated for the first time that addition of Ba^{2+} causes relaxation of K^{+} -depolarized canine coronary arteries in the presence of diltiazem. This relaxation was inhibited by removal of endothelial cells or by L-NMMA and L-NNA, both of which are inhibitors of NO synthase (13). Furthermore, addition of divalent cations increased the cyclic GMP levels in endothelium-intact arteries but not those in endothelium-denuded arteries. Therefore, synthesis of NO in endothelial cells is in part responsible for the Ba^{2+} - and Sr^{2+} -induced relaxation of K^{+} -depolarized canine coronary arteries.

However, there were some differences in NO-dependent relaxation and cGMP production between the three cations, although they are all in the group of alkaline earth cations. Ba^{2+} - and Sr^{2+} -induced relaxation was inhibited by NO synthase inhibitors to about half the control value, while Ca^{2+} -induced relaxation was completely inhibited by these agonists. Nevertheless, all of these cations increased the cyclic GMP level to a similar extent, and this effect was completely inhibited by L-NMMA. These results indicate that Ca^{2+} -induced relaxation is almost totally dependent on the NO pathway, whereas Ba^{2+} - and Sr^{2+} -induced relaxation is largely, but not exclusively, dependent on this pathway.

Fig. 3. Effects of L-NNA on Ca^{2+} -, Ba^{2+} - and Sr^{2+} -induced relaxation of canine coronary artery in the presence or absence of a combination of propranolol, phentolamine and atropine. The arteries were suspended in K^{+} (80 mM)-depolarizing, Ca^{2+} -free PSS. Ca^{2+} (a), Ba^{2+} (b) or Sr^{2+} (c) (10^{-3} M) was applied 10 min after addition of diltiazem (3×10^{-6} M) in the absence (circles) or presence (squares) of a combination of propranolol, phentolamine and atropine (each 10^{-6} M). The combination of these antagonists was applied 15 min before the addition of each cation. \circ , \square : Control, \bullet , \blacksquare : L-NNA (3×10^{-5} M in panel a and 3×10^{-4} M in panels b and c). Percentage relaxation relative to the papaverine (10^{-4} M)-induced relaxation is shown on the ordinate. Each agent was applied as indicated by the bar. Each point represents the mean of 3–6 experiments with S.E. * $P < 0.05$, ** $P < 0.01$, compared with the control group. # $P < 0.05$, ## $P < 0.01$, compared with the group in the absence of propranolol, phentolamine and atropine.

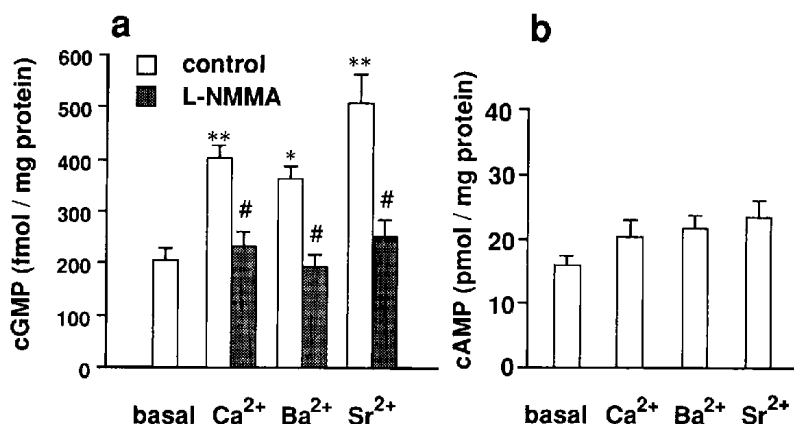


Fig. 4. Effects of divalent cations (10^{-3} M for 5 min) on tissue levels of cyclic GMP (a) and cyclic AMP (b) in endothelium-intact rings of canine coronary artery in the presence of diltiazem (3×10^{-6} M). The arteries were incubated in K^{+} (80 mM)-depolarizing, Ca^{2+} -free PSS. L-NMMA (3×10^{-4} M) was applied 5 min before the addition of each cation. Each point represents the mean of 5–9 experiments with S.E. * $P < 0.05$, ** $P < 0.01$, compared with each basal level. # $P < 0.05$, compared with the corresponding control for each cation.

The Ca^{2+} -, Ba^{2+} - and Sr^{2+} -induced relaxation was not inhibited by indomethacin, an inhibitor of cyclooxygenase. Furthermore, addition of these cations did not affect cyclic AMP levels. Thus, synthesis of prostacyclin as a mechanism for the relaxation could be excluded. Feletou and Vanhoutte (14) have suggested that another endothelium-derived relaxing substance, named endothelium-dependent hyperpolarizing factor (EDHF) (15), causes relaxation through hyperpolarization of the canine coronary artery. However, EDHF was not responsible for these cation-induced relaxations in this study because we used K^{+} (80 mM)-depolarized arteries.

Role of neurotransmitters

Functional and histochemical studies have shown that adrenergic and cholinergic fibers innervate coronary arteries and that this mechanism is involved in the relaxation induced by electrical stimulation (16, 17). These fibers seem to have a role in Ca^{2+} -, Ba^{2+} - and Sr^{2+} -induced relaxation. According to Nakazato and Onoda (18), Ba^{2+} and Sr^{2+} can substitute for Ca^{2+} in noradrenaline release induced by excess potassium in the vas deferens of the guinea pig. In the present study, Ca^{2+} -, Ba^{2+} - and Sr^{2+} -induced relaxation was attenuated by a combination of antagonists of β - and α -adrenoceptors and muscarinic receptors. Therefore, neurotransmitters released by these cations in part contribute to the relaxation of the coronary arteries. This may explain the small degree of relaxation that was observed in the endothelium-denuded artery as shown in Fig. 1. In the case of Ca^{2+} , these fibers have an apparently minor role, since relaxation was overcome by treatment with L-NMMA and L-NNA in the absence of the antagonist combination.

In contrast to the situation with Ca^{2+} , approximately 20% of the Ba^{2+} - and Sr^{2+} -induced relaxation was maintained in coronary arteries treated with L-NNA in the presence of adrenergic and cholinergic antagonists, although the increase in cyclic GMP levels was completely blocked by L-NMMA. This may be due to differences in the extent of contraction of K^{+} -depolarized coronary arteries induced by each cation, because the pEC_{50} from the concentration-response curve for Ca^{2+} was greater than those for Ba^{2+} and Sr^{2+} .

Differences in responses to divalent cations

Both Ca^{2+} and calmodulin have been shown to be required for the constitutive NO synthase in the endothelium (4, 5, 19–21). Thus, the affinity of divalent cations for calmodulin may be important for the activation of NO synthase. Ca^{2+} and Sr^{2+} have been shown to evoke the tyrosine fluorescence of calmodulin maximally at concentrations of approximately 7 moles Ca^{2+} and 40 moles Sr^{2+} per mole calmodulin (22). Therefore, Sr^{2+} may easily substitute for Ca^{2+} in the activation of calmodulin. However, Ba^{2+} has been reported to be completely ineffective in inducing the tyrosine fluorescence of calmodulin even at concentrations of 50 moles Ba^{2+} per mole calmodulin (22).

It remains unclear how Ba^{2+} is able to stimulate EDRF/NO release. One explanation is that Ba^{2+} itself may accumulate in endothelial cells at levels sufficient to compensate for the low affinity of Ba^{2+} for calmodulin, since it is not taken up into intracellular stores or readily removed from the cells. This explanation has also been discussed in previous studies concerning the calmodulin-dependent contraction of smooth muscle (23, 24) and

Ca²⁺-ATPase in human erythrocytes (25). Another possibility is that Ba²⁺ may induce the release of Ca²⁺ from endothelial intracellular Ca²⁺ stores, as has been proposed for Ba²⁺-induced contraction in smooth muscle (24, 26). To determine the exact reason, further studies are needed on the relationship between Ba²⁺ concentrations and Ba²⁺-induced activation of NO synthase in endothelial cells.

Active tone in depolarized coronary artery

Under the conditions we used, depolarized canine coronary arteries possessed some active tone even in nominally Ca²⁺-free, K⁺-depolarizing PSS, since their tone was reduced by a NO-releasing agent, an adrenoceptor agonist and a smooth muscle relaxant. Extracellular Ca²⁺ is likely to contribute only partially to this active tone, since the basal tension remained after EGTA was applied. Thus, intracellular Ca²⁺ stores may play a role in generating this active tone. Although diltiazem did not decrease the basal tension in the present study, a much higher concentration than that necessary to inhibit the contraction evoked by the release of sequestered Ca²⁺ (27) might change the active tone. The exact mechanism underlying this tone requires further clarification, but it provides a useful bioassay system for the investigation of divalent cation-induced relaxation.

The present study has revealed that endothelial function can be regulated by Ba²⁺. Although the physiological relevance of this fact is uncertain, this study raises interesting questions such as what is the mechanism by which Ba²⁺ activates endothelial NO synthase and what is the concentration of Ba²⁺ necessary for its endothelial function? In conclusion, Ba²⁺ and Sr²⁺ were found to substitute for Ca²⁺ in producing NO in the endothelium and to relax canine depolarized coronary arteries through cGMP production.

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