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The crucial role of physiological Ca^{2+} concentrations in the production of endothelial nitric oxide and the control of vascular tone

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1 The effect of varying the extracellular Ca^{2+} concentration on the basal and acetylcholine (ACh)-induced release of nitric oxide (NO) from the rabbit aorta was investigated by use of a superfusion bioassay system.

2 Changes between 0.5 and 2.0 mM in the concentration of Ca^{2+} superfusing the detector bioassay tissues or perfusing endothelium-denuded donor aortae had no effect on the tone of these tissues.

3 Increasing the concentration of Ca^{2+} perfusing endothelium-containing donor aortae from zero to 1.25 mM caused a transient (24 ± 9 min), concentration-dependent basal release of NO, which was attenuated at higher concentrations of Ca^{2+} (1.5–2.0 mM).

4 The duration of the effect of Ca^{2+} on the basal release of NO was increased by a concomitant infusion of L-arginine (100 μM) through the donor aorta.

5 Changes in the concentration of Ca^{2+} between 0.5 and 2.0 mM had a similar biphasic effect on the release of NO induced by ACh, which was also maximal at 1.25 mM Ca^{2+}.

6 When Ca^{2+} was removed from the Krebs buffer perfusing the donor aorta, the basal release of NO declined within 2 min. In contrast, the release of NO induced by ACh declined progressively over 60 min.

7 Thus changes in the concentration of Ca^{2+} around the physiological range modulate the synthesis of NO by the vascular endothelium and consequently, vascular tone. This may account for the effects of dietary Ca^{2+} supplements on the control of some hypertensive states.

Introduction

The vascular endothelium produces nitric oxide (NO) (Palmer *et al.*, 1987) which is synthesized from the terminal guanidino nitrogen atom(s) of L-arginine (Palmer *et al.*, 1988a). Furthermore, this synthesis is inhibited by the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA; Palmer *et al.*, 1988b; Rees *et al.*, 1989a). The synthesis of NO by the vascular endothelium plays a significant role in the control of blood pressure (Rees *et al.*, 1989b) and flow in animals (Gardiner *et al.*, 1990) and man (Vallance *et al.*, 1989), This has led to the suggestion that the generation of NO from L-arginine maintains a vasodilator tone in the cardiovascular system (Rees *et al.*, 1989b; Moncada & Palmer, 1990).

The release of NO accounts for the biological actions of endothelium-derived relaxing factor (EDRF; for review see Moncada *et al.*, 1988). The release of EDRF and the endothelium-dependent vascular relaxation are dependent on transmembrane Ca^{2+} flux rather than on mobilization of intracellular Ca^{2+} stores (Luckhoff *et al.*, 1988; White & Martin, 1989). In addition, the endothelial NO synthase is a Ca^{2+}-dependent enzyme (Palmer & Moncada, 1989; Moncada & Palmer, 1990; Meyer *et al.*, 1989).

Epidemiological studies have shown that restricted dietary Ca^{2+} is associated with elevations in blood pressure (McCarron *et al.*, 1982; Kesteloot & Joossens, 1988), and with increased incidence of pregnancy-induced hypertension (PIH; Belizan & Villar, 1980). Studies with animal models of hypertension have verified this association (Ayachi, 1979; Peuler *et al.*, 1987) and have further suggested that increases in dietary Ca^{2+} lower the blood pressure (Furspan *et al.*, 1989; Hatton *et al.*, 1989). Clinical trials have confirmed that dietary Ca^{2+} supplements can lower blood pressure in some hypertensive individuals (Grobbee & Hofman, 1986) and in normotensive pregnant women (Lopez-Jaramillo *et al.*, 1989) and can also reduce the incidence of PIH (Lopez-Jaramillo *et al.*, 1990). The beneficial effect of Ca^{2+} supplementation was more evident in animal models of hypertension (McCarron *et al.*, 1981) and in those essential hypertensive patients (Resnick *et al.*, 1986) and pregnant women (Lopez-Jaramillo *et al.*, 1989) whose ionised Ca^{2+} concentration in serum was low, but which could be raised by Ca^{2+} supplementation.

Several hypotheses have been proposed to explain the mechanisms by which Ca^{2+} supplementation acts to reduce blood pressure; these include changes in smooth muscle membrane stability (Furspan & Bohr, 1986), natriuresis and associated changes in sodium homeostasis (Zemel *et al.*, 1988), depletion of phosphate (Lau *et al.*, 1984) and increases in prostacyclin synthesis (Lopez-Jaramillo *et al.*, 1987a,b). To date, however, none of these have been substantiated.

Because of all this we decided to investigate whether small variations in extracellular Ca^{2+} concentration around the physiological range alter the production of NO by the vascular endothelium and are, therefore, relevant to the understanding of the physiological regulation of vascular tone and its changes during conditions such as hypertension and PIH.

Methods

Bioassay of NO

Male New Zealand White rabbits (2.0–2.3 kg) were killed with an overdose of sodium pentobarbitone. A segment (6 cm) of the thoracic aorta was removed, trimmed free of adhering fat and connective tissue and placed in a perspex chamber. The tissue was then perfused intraluminally at 5 ml min^{-1}, by a Watson Marlow 101 U/R pump, with Krebs buffer, gassed with 95% O_2/5% CO_2 at 37°C, containing indomethacin

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(5 μM) and different concentrations of Ca^{2+} (0.5, 0.75, 1.0, 1.25, 1.5 and 2.0 mM). This aorta was called the donor tissue. In some experiments this donor tissue was denuded of endothelium by gently rubbing the endothelial surface with a pipe cleaner as described previously (Rees *et al.*, 1989a).

For the bioassay, the effluent of the donor tissue was used to superfuse two spiral strips of rabbit thoracic aorta, denuded of endothelium (bioassay tissues), in a cascade as described previously (Rees *et al.*, 1989a). The Ca^{2+} concentration in the fluid superfusing these tissues was kept constant by mixing the effluent of the donor tissue with sufficient Ca^{2+} to maintain a concentration of 2 mM. After a period of 40 min equilibration, the bioassay tissues were contracted with a continuous infusion of 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619; 50 nM). Atropine sulphate (0.2 μM) was continuously infused over the bioassay tissues (O.T.) in order to block any direct action of acetylcholine (ACh).

The tension of bioassay tissues was recorded with Harvard Bioscience isotonic transducers on a Linearcorder mark VII WR3101 (Graphtec). The amplification of the recorder was adjusted so that similar relaxations to glyceryl trinitrate (GTN; 20 nM) were observed in each bioassay tissue. In some experiments, L-arginine (100 μM), haemoglobin (Hb, 3.5, 6.25 nM), superoxide dismutase (SOD; 15 u ml^{-1}), L-NMMA (100 μM), D-NMMA (100 μM) or N-iminoethyl-L-ornithine (L-NIO; 100 μM) were added to the perfusate at a rate of 100 μl min^{-1}. The release of an unstable rabbit aorta-relaxing substance which could be potentiated by SOD and inhibited by Hb and by arginine analogues, which are known inhibitors of NO synthesis (Rees *et al.*, 1989a), was identified as NO.

The release of NO was stimulated in some experiments by ACh (1 μM), which was delivered as a 1 min infusion through an injection port immediately before the donor tissue. Exogenous NO (220 pmol) was given as a bolus injection over the bioassay tissues.

Materials

Nitric oxide (>99.98 pure, British Oxygen Corporation); $CaCl_2$ (BDH Analar), glyceryl trinitrate (Wellcome), indomethacin, ACh, SOD, atropine sulphate, L-arginine free base, Hb (Sigma), sodium pentobarbitone (May & Baker) and U46619 (Cayman Chemical) were obtained as indicated. L-NMMA, D-NMMA and L-NIO were synthesised by Dr H.F. Hodson of Wellcome Research Laboratories. All drugs used were administered in saline solution.

Statistics

Results are expressed as mean ± s.e.mean for n separate experiments. One way analysis of variance (ANOVA) was used to examine differences between the mean of the relaxation at the different Ca^{2+} concentrations. Contractions or relaxations of the bioassay tissues are expressed as % of the relaxation obtained in the uppermost bioassay tissue with a standard (20 nM) bolus injection of GTN.

Results

Effect of Ca^{2+} on basal release of NO

Changes in Ca^{2+} concentrations between 0.5 and 2 mM did not have a direct effect on the tone of the bioassay tissues, either when they were without tone, or when contracted with U46619 (n = 3 for each). In addition they had no effect on the actions of NO administered directly O.T. (n = 3). When the bioassay tissues were superfused with Ca^{2+}-free Krebs solution for longer than 1 h, a small concentration-independent contraction was observed with the first addition of Ca^{2+} (n = 5). No further change in the tone of the bioassay tissues was observed with subsequent addition of Ca^{2+} (0.5–2.0 mM). Furthermore, if the concentration of Ca^{2+} was changed between 0.5–2.0 mM in a donor tissue denuded of endothelium, there was no change in the tone of the bioassay tissues (n = 3).

When the concentration of Ca^{2+} perfusing a donor aorta with intact endothelium was changed from 2.0 to 1.25 mM, the release of an EDRF-like substance was observed (Figure 1). This substance was unstable, its stability was enhanced by SOD (15 u ml^{-1}; n = 3; data not shown). Furthermore, its action was antagonized by Hb (3.5, 6.25 nM; n = 3; Figure 1). Moreover, infusion of L-NMMA (100 μM) or L-NIO (100 μM), but not D-NMMA (100 μM), also abolished the release of NO (n = 3 for each). These results indicated that this EDRF-like substance was NO.

The release of NO which also occurred when the Ca^{2+} concentration was changed from zero to 1.25 mM resulted in the relaxation of the uppermost bioassay tissue, the tone of which was reduced by 51.4 ± 6.4% (n = 17) of that induced by a standard dose of GTN (20 nM). When the Ca^{2+} concentration was further increased to 1.5–2.0 mM, release of NO was reduced, such that the tone of the bioassay tissue was 7.9 ± 5.1% (n = 7) of the standard GTN dose.

The basal release of NO observed when the Ca^{2+} concentration perfusing the donor tissue was changed from zero to 1.25 mM was further increased when the donor tissue was perfused with L-arginine (100 μM), such that the relaxation of the uppermost bioassay tissue was 31.5 ± 18% (n = 5) greater than that obtained with 1.25 mM Ca^{2+} alone (Figure 2). Moreover, L-arginine increased the duration of the Ca^{2+}-induced release of NO from 24 ± 9 min to 42.5 ± 4 min (n = 5).

When Ca^{2+} was removed from the Krebs buffer perfusing the donor tissue, the tone of the uppermost bioassay tissue increased significantly compared to that in the presence of 1.25 mM Ca^{2+} (Figure 3).

Effect of Ca^{2+} on NO release induced by ACh

The release of NO induced by ACh (1 μM) was also dependent on the extracellular Ca^{2+} concentration. When Ca^{2+} was removed from the Krebs solution, the release of NO induced by ACh was progressively reduced and was abolished following perfusion of the donor tissue for 60 min in Ca^{2+}-free Krebs solution (Figure 3).

The release of NO induced by ACh was maximal in the presence of 1.25 mM Ca^{2+} (Figure 4). At 1.0 mM Ca^{2+} the release was reduced, but was not significantly different from that in the presence of 1.25 mM Ca^{2+}. At higher (1.5, 2.0 mM) or lower (0.5, 0.75 mM) Ca^{2+} concentrations, the release of NO

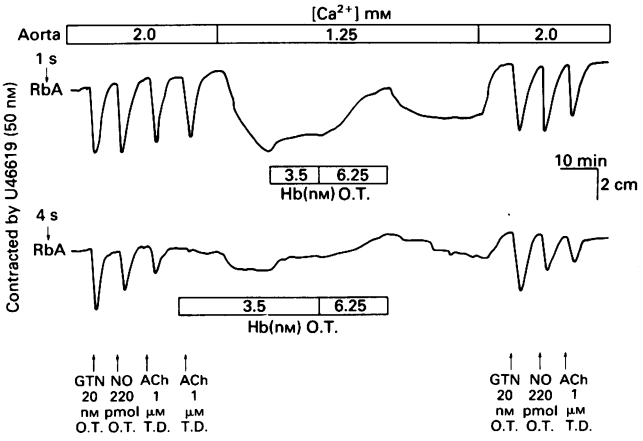

Figure 1 Effect of haemoglobin (Hb) on the action of nitric oxide (NO). Infusion of haemoglobin (3.5 or 6.25 nM) over the bioassay tissues (O.T.) inhibited the relaxation induced by NO released both by acetylcholine (ACh, 1 μM) and by decreasing the concentration of Ca^{2+} perfusing the donor aorta from 2.0 mM to 1.25 mM. Trace representative of 3 similar experiments. RbA = rabbit aorta, GTN = glyceryl trinitrate, T.D. = applied to the donor tissue.

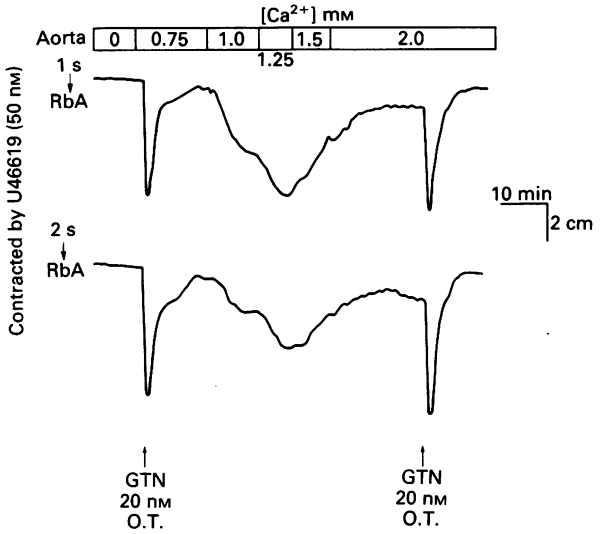

Figure 2 Effect of Ca^{2+} concentration on basal release of nitric oxide (NO) in the presence of 100 μM L-arginine. When the Ca^{2+} concentration perfusing a donor aorta was increased to 1.0 mM, the release of NO was observed and this was enhanced when the Ca^{2+} concentration was increased to 1.25 mM. When the Ca^{2+} concentration was further increased (1.5–2.0 mM), a decline in the release of NO was observed. Trace representative of 5 similar experiments. For key to abbreviations used see legend of Figure 1.

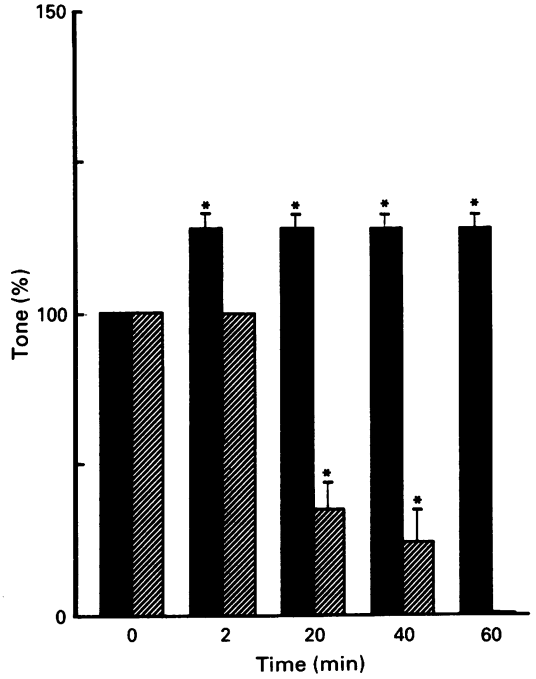

Figure 3 Change in tone of the bioassay tissues (solid columns) and in the relaxing response (hatched columns) to acetylcholine (ACh) with time following a change in Ca^{2+} concentration from 1.25 mM to zero. A significant increase in the tone of the uppermost bioassay tissue was observed 2 min after the removal of the Ca^{2+} in the donor tissue perfusate. The release of nitric oxide (NO) induced by ACh was progressively reduced following perfusion of the donor aorta for 60 min in Ca^{2+}-free Krebs solution. Changes in the basal tone of the uppermost bioassay tissue were expressed as a % of the relaxation induced by a standard dose of glyceryl trinitrate (GTN, 20 nM). Changes in the release of NO induced by ACh were expressed as a % of that in the presence of 1.25 mM Ca^{2+}. Each value is the mean of 5 separate determinations; bars show s.e.mean. * Indicates that the value differs significantly from the value observed at 1.25 mM Ca^{2+}.

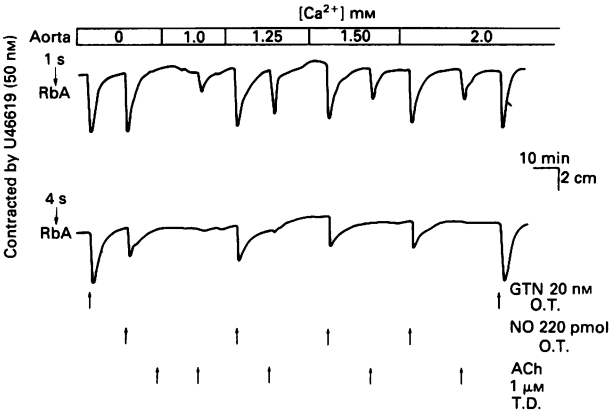

Figure 4 Effect of Ca^{2+} concentration on nitric oxide (NO) release induced by acetylcholine (ACh, 1 μM). The maximum release was observed when the Ca^{2+} concentration was 1.25 mM. At higher or lower Ca^{2+} concentrations, there was a significant reduction in NO release. Trace representative of 7 similar experiments. For key to abbreviations used see legend of Figure 1.

was significantly ($P < 0.05$) reduced compared to that in the presence of 1.25 mM Ca^{2+} (Figure 5).

Discussion

The present study confirms that the synthesis of endothelial NO is critically dependent on the extracellular concentration of Ca^{2+} and demonstrates that small changes in the concentration of this cation around the physiological range fundamentally affect NO release.

When donor aortae with intact endothelium were perfused with Krebs buffer containing Ca^{2+} concentrations within the physiological range, basal release of an EDRF-like substance was observed. At Ca^{2+} concentrations outside the physiological range, both above and below, there was a decline in this basal release. The vascular relaxing activity released was inhibited by Hb and by the inhibitors of NO synthase, L-NMMA and L-NIO, but not by D-NMMA, and was potentiated by SOD. These are all characteristics of NO (for review see Moncada *et al.*, 1988). Changes in Ca^{2+} concentration over the range used in the present study had no effect on the tone of the bioassay tissues, either under basal conditions or when contracted with U46619, and did not induce the release of a relaxing substance from endothelium-denuded donor

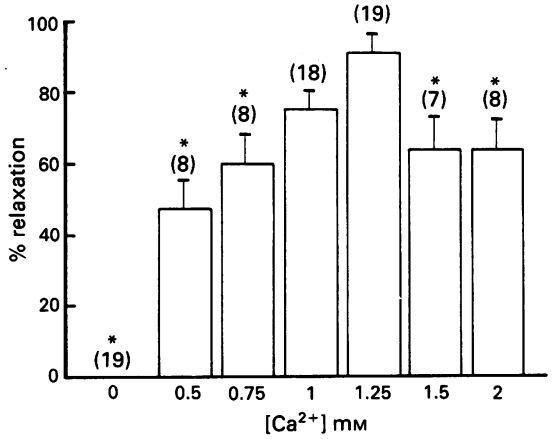

Figure 5 Relaxation of the uppermost bioassay tissue by nitric oxide (NO) released by acetylcholine (ACh, 1 μM). The donor tissue was perfused with Krebs solution containing different Ca^{2+} concentrations. Each value is the mean and bars show s.e.mean of n (number in parentheses) separate determinations. * Indicates that the value differs significantly from the value observed at 1.25 mM Ca^{2+}.

aortae, confirming that the endothelium is the site of action of Ca^{2+}. Since the endothelial NO synthase is Ca^{2+}-dependent (Meyer *et al.*, 1989; Moncada & Palmer, 1990), it is likely that these affects of Ca^{2+} are mediated by modulating the activity of this enzyme.

The magnitude of basal release of NO in the absence of extracellular Ca^{2+} cannot be determined by the present bioassay technique. However, Luckhoff *et al.* (1988) did not observe detectable NO release in the absence of extracellular Ca^{2+} using the activation of a soluble guanylate cyclase to detect NO. These findings suggest that the modulation of basal NO release by physiological Ca^{2+} concentrations will have a considerable effect on the resultant vascular tone. Furthermore, they indicate that in the majority of studies *in vitro*, which are carried out in the presence of 2.5 mM Ca^{2+}, the release of NO is not being studied under optimum conditions.

The mechanism whereby concentrations of Ca^{2+} above the physiological range attenuate the basal release of NO is not clear. It is possible that, as in the cerebellum (Knowles *et al.*, 1989), high Ca^{2+} concentrations might down-regulate the guanylate cyclase in the vascular smooth muscle of the detector tissues. This does not seem to be the case in smooth muscle cells, since the response of the bioassay tissues to NO was not affected by the Ca^{2+} concentration.

The release of NO by physiological Ca^{2+} concentrations was transient, but its duration could be significantly enhanced by exogenous L-arginine. These data indicate that, in addition to the concentration of Ca^{2+}, the availability of L-arginine contributes to the regulation of NO synthesis by the vascular endothelium.

The release of NO induced by ACh was also dependent on the extracellular Ca^{2+} concentration, being maximal in the physiological range and abolished in the absence of Ca^{2+}. Interestingly, the release of NO by ACh was observed at both low (0.5–0.75 mM) and high (1.5–2.0 mM) Ca^{2+} concentrations, conditions under which basal release of NO was not detectable. This suggests either that there are different mechanisms whereby Ca^{2+} enters the cell to stimulate the synthesis of NO, or that ACh is a more effective stimulus which can rely to some extent on intracellular Ca^{2+}. This suggestion is reinforced by the finding that, following transfer from physiological (1.25 mM) to zero Ca^{2+} concentration, the basal release of NO declines much more rapidly than the stimulated release.

At present, the physiological stimuli for the release of NO are not known. Mechanical factors, such as shear stress (Pohl *et al.*, 1986) and pulsatile flow (Rubanyi *et al.*, 1986), are postulated to be major stimuli for the release of NO. The entry of Ca^{2+} into endothelial cells may occur through channels that open in response to 'stretch' (Lansman *et al.*, 1987). Furthermore, Ando *et al.* (1988) have demonstrated an increase in cytosolic free Ca^{2+} levels in bovine aortic endothelial cells in culture subjected to controlled degrees of shear stress. It is likely, therefore, that the basal release of NO occurs as a consequence of the opening of this stretch-activated channel by the pulsatile flow used in our system. Furthermore, since ACh activates receptor-operated Ca^{2+} channels (Danthuluri *et al.*, 1988), it is likely that the consequences of activation of these two routes of Ca^{2+} entry into cells accounts for the differential effect of Ca^{2+} on basal and stimulated release of NO.

A physiological role for endothelium-derived NO in the control of vascular tone *in vivo* has recently been demonstrated in animals and man. Intravenous administration of L-NMMA induced a dose-dependent rise in blood pressure in animals (Rees *et al.*, 1989b; Whittle *et al.*, 1989; Gardiner *et al.*, 1990) and had a vasoconstrictor effect in the arterial system of the human forearm (Vallance *et al.*, 1989).

Individuals with essential hypertension tend to have low ionised Ca^{2+} concentrations in serum (McCarron, 1982; Resnick, 1987). Dietary Ca^{2+} supplementation trials in hypertensive populations have yielded variable results, although anti-hypertensive effects are seen in those subgroups of the hypertensive population who exhibit low ionised Ca^{2+} concentration in serum (Grobbee & Hofman, 1986; Resnick *et al.*, 1986). A decrease in serum ionised Ca^{2+} occurs during pregnancy (Pitkin & Gebhardt, 1977; Lopez-Jaramillo *et al.*, 1988). In pregnant women, dietary Ca^{2+} supplementation maintains the Ca^{2+} levels in the physiological range (Lopez-Jaramillo *et al.*, 1989) and significantly reduces blood pressure (Villar *et al.*, 1987; Marya *et al.*, 1987) and the incidence of PIH (Lopez-Jaramillo *et al.*, 1990). A similar effect of higher dietary Ca^{2+} has also been observed in the spontaneously hypertensive rat, but not Wistar-Kyoto rats. In these animals, Ca^{2+} supplementation increased serum ionised Ca^{2+} (Furspan *et al.*, 1989) and decreased blood pressure (Ayachi, 1979; Furspan *et al.*, 1989; McCarron *et al.*, 1981).

In summary, our results indicate that Ca^{2+} plays a crucial role in the modulation of vascular tone. The effect is mediated by the regulation of the synthesis of endothelial NO rather than by a direct effect on vascular smooth muscle. These findings may explain the paradoxical action of increased serum Ca^{2+} and the antihypertensive effect observed in some hypertensive states (McCarron *et al.*, 1987; Zaloga *et al.*, 1988; Furspan *et al.*, 1989). Furthermore, they suggest that small variations in the extracellular Ca^{2+} levels may be of relevance in the development of some forms of hypertension including PIH.

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