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Role of Endogenous Nitric Oxide in the Vasorelaxation Induced by High Calcium Environment *in vitro*

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=ABSTRACT=

The present study was undertaken to examine if endogenous nitric oxide is partly responsible for the high calcium-induced vasorelaxation *in vitro*. Isolated porcine coronary arterial rings were suspended in the tissue chamber and their changes in isometric tension were recorded. KCl little affected the vascular tension in the calcium-free media, but subsequent addition of cumulative doses of $CaCl_2$ from 1 to 40 mM caused a contraction followed by complete relaxation. The maximum tension was noted at the calcium concentration in the media of 5 mM, and then the tension progressively declined at 10-40 mM. The relaxation was slightly attenuated in the endothelium-denuded preparation. The relaxation was converted into a contraction by the addition of methylene blue. The relaxation response was not affected in the presence of indomethacin, but was significantly attenuated by N^{ω}-nitro-L-arginine methyl ester pretreatment. These results suggest that the calcium-induced vasorelaxation is in part attributable to the release of endogenous nitric oxide.

Key Words: Porcine coronary artery, High calcium media, Vasorelaxation, N^{ω}-nitro-L-arginine methyl ester, Endogenous nitric oxide.

INTRODUCTION

As early as at the dawn of this century, Douglas Cow (1911) observed that excess calcium in the muscle bath depressed the contractile response of sheep splenic arterial rings to noradrenaline and pituitrin. Since then, the ability of elevated external calcium to reduce the contractile response of vascular smooth muscle has been variously documented.

Johnson et al (1986) observed changes in vascular tension as a function of calcium concentration in the bathing media using porcine coronary artery. With increasing calcium concentrations, they found that the tension increased (vasoconstriction) only until the concentration reached certain limit and thereafter declined (vasorelaxation). As has been suggested by the same authors, membrane stabilization due to calcium channel blockade may be mainly responsible for the vasorelaxation. The additional mechanism(s) by which the high calcium induces vasorelaxation have not been established, however.

Recent interest on the role played by the endothelium in regulating the vascular tone has focused on its synthesis and secretion of various vasoactive products. Among them are two unstable vasodilators, prostacyclin (Moncada et al, 1976) and endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980).

EDRF has now been characterized as nitric oxide (Palmer et al, 1987; Ignarro et al, 1987). Indeed, as with other nitrodilators, EDRF can

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evoke relaxation in association with increases in intracellular cGMP levels of vascular smooth muscle cells (Ignarro, 1989) and consequent inactivation of the contractile protein, implying activation of soluble guanylyl cyclase and cGMP cascade (Murad, 1986).

A role for calcium in the synthesis and release of nitric oxide has recently been demonstrated (Luckhoff et al, 1988). In this context, Wu and Bohr (1991) have suggested that the calcium-induced relaxation is associated with the role of intact endothelium and production of EDRF, although evidence is not clear.

On the other hand, it has been found that synthesis of nitric oxide can be antagonized by some compounds such as N^{ω}-monomethyl-L-arginine methyl ester (Ishii et al, 1990). Therefore, if calcium relaxation is due to a release of endogenous nitric oxide, it may be attenuated by such a treatment.

The purpose of the present study was to examine the hypothesis that endogenous nitric oxide is partly responsible for the calcium-induced vasorelaxation *in vitro*.

MATERIALS AND METHODS

Porcine hearts were obtained from a local abattoir and immersed in cold physiological salt solution (PSS). The right coronary artery was removed and trimmed clean of connective and adipose tissue. The artery was then cut into rings, 5-mm long each. Endothelium-denuded (-EC) preparations were made by gentle rubbing of the intimal layers with a metal rod inserted into the lumen of the artery. Each ring was suspended in a tissue bath containing PSS at 37 ± 0.5°C, and being continuously bubbled with 95 % O_2-5% CO_2 (pH 7.35). Baseline load placed on the ring was 2.0 g, and its isometric tension was recorded using force-displacement transducer (Grass FT03). The rings were equilibrated for 2 h before the experiment begun. The composition of the PSS used was as follows (in mM): NaCl 112, KCl 5, $NaHCO_3$ 15, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 11.5.

Drugs used were methylene blue, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and indomethacin, purchased from Sigma Chemical Company. The stock solutions were made in distilled water except indomethacin which was dissolved in methanol. The methanol concentration in the bath did not exceed 0.01 %, at which contractile or relaxation response was not affected.

Contraction and relaxation were calculated as percent of the maximum contraction attained. Results were expressed as means ± SE. Statistical significance was determined using paired *t*-test where the arterial rings taken from the same animal were compared.

RESULTS

Effects of calcium on the vascular tension

The arterial ring was little affected by the addition of KCl (30 mM) in the calcium-free media. As calcium concentration cumulatively increased in the media, the vascular tension increased until the concentration reached 5 mM. The tension then progressively declined: at 20 mM it diminished to a one fifth of the maximum and at 40 mM to virtually zero (Fig. 1 & 2).

The same concentration changes induced by $BaCl_2$ produced an increase of tension without subsequent relaxation (Fig. 1). In addition, the maximum contraction attained by calcium (5 mM) was not affected by subsequent addition of sucrose (10, 20, 40 mM), of which molar concentrations were the same as those of $CaCl_2$.

The osmolalities in the media following administration of $CaCl_2$, $BaCl_2$ and sucrose up to 40 mM each were comparable: 392 ± 7 (n = 6), 421 ± 12 (n = 7) and 407 ± 16 (n = 6) mOsm/kg H_2O, respectively.

Effects of endothelium removal or L-NAME treatment on the calcium-induced vasorelaxation

The magnitude of relaxation was only slightly but significantly attenuated in the -EC preparations compared with the control (Fig. 2). Pretreatment of the media with L-NAME

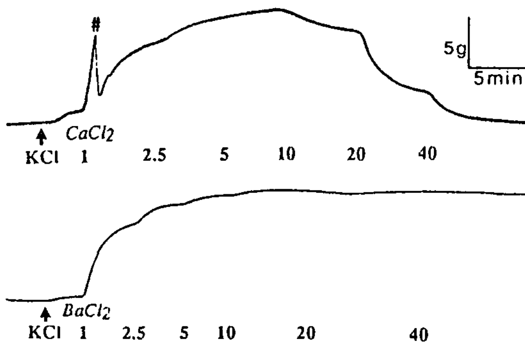
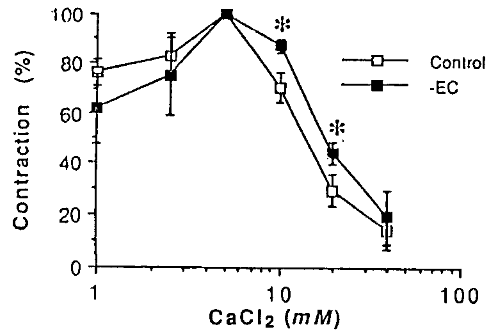


Fig. 1. Effects of cumulative doses of calcium and barium on the KCl-depolarized coronary arterial ring. At arrow marks, KCl was added (30 mM), and the following numerals indicate the concentrations of $CaCl_2$ or $BaCl_2$ in mM. At #, the gain was reduced half.



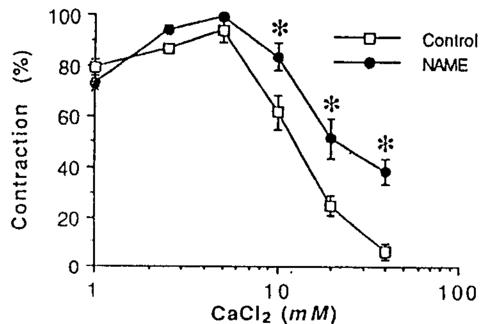


*Fig. 2. Effects of endothelium-removal and L-NAME on the vascular responses to the calcium. The maximum tension attained was regarded as 100%. - EC: Endothelium-denuded preparation. NAME: L-NAME was treated (10^{-4} M) 30 min prior to KCl. Each point represents the mean of 5-9 rings. *p<0.01, compared with the control.*

(10^{-4} M) significantly attenuated the relaxation response (Fig. 2). The maximal tensions attained were 7.9±0.4 (n=22), 11.4±0.8 (n=7) and 11.2±0.5 g (n=14) in control, -EC, and L-NAME treated groups, respectively, the latter two being significantly higher than the control (non-paired t-test, $p<0.01$).

Effects of indomethacin and methylene blue on the relaxation

Indomethacin pretreatment (10^{-6} M) reduced the maximum tension attained by the calcium (4.5±0.5 g, n=6) without affecting the magnitude of subsequent relaxation (data not shown). Methylene blue (10^{-5} M), of which concentration *per se* did not cause any contraction, converted the calcium-relaxation into a contraction (Fig. 3).

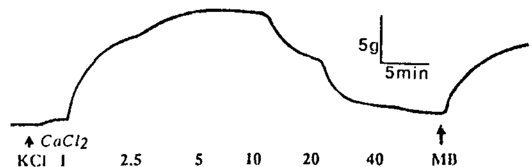


Fig. 3. Effects of methylene blue on the vascular relaxation induced by calcium. At MB, methylene blue was added (10^{-5} M). Other legends as in Fig. 1.

DISCUSSION

This and the previous (Johnson et al, 1986) studies coincided in that pig coronary artery showed a relaxation following the initial contraction as the calcium concentration in the media was progressively increased. Johnson et al (1986) have suggested that the calcium binds

to a calcium binding protein on the channel, resulting in the channel blockade to prevent further influx of calcium and relaxation.

Apart from this membrane stabilization due to high calcium, the slightly attenuated relaxation in the endothelium-denuded rings suggests a functional association of the endothelium with the relaxation.

Furthermore, a partial restoration of tension by methylene blue suggests that the relaxation was partly mediated by the cGMP system. Similarly, Wu and Bohr (1991) observed that methylene blue pretreatment partially blocked the relaxation, and concluded that the relaxation was mediated by releasing EDRF and cGMP. The diminished relaxation in the presence of L-NAME, nitric oxide synthase inhibitor, in the present study, further supports the hypothesis that endogenous nitric oxide mediates the relaxation. These results also may rule out a possible intervention of endothelelium-derived hyperpolarizing factor for the relaxation, since this does not act by increasing cGMP (Taylor et al, 1988).

Nitric oxide, via cGMP as a second messenger, enhances calcium entry into the sarcoplasmic reticulum (Lincoln, 1983) and inhibits receptor-operated calcium channel (Godfraind, 1986), both mechanisms resulting in decreases of intracellular calcium level. Taken together, high calcium environment may have a dual effect: one directly on the membrane calcium channel, and the other in a mediation through release of endogenous nitric oxide, both culminating in decreases of intracellular calcium concentration and vasorelaxation.

It has been suggested that both the stimulated and the background release of EDRF are calcium-dependent (Spedding et al, 1986), and the source of calcium to be mainly extracellular (Long & Stone, 1985). Therefore, one may argue that the source of calcium to elicit the release of EDRF from the endothelium is unclear in the present study.

Although many investigators agree that endothelial cells do not have voltage-dependent calcium channels (Campbell et al, 1991; Takeda et al, 1987), however, the role of a calcium channel may not be completely ruled out. Slow calcium channel blockers (verapamil and nifedipine) partially inhibits the release of EDRF (Singer & Peach, 1982). Williams et al (1987) also have suggested that porcine coronary endothelial cells may contain voltage-dependent calcium channels. In addition, Doler et al (1992) have recently suggested that extracellular calcium enters the cell through an activated cation entry pathway, although its implication in EDRF release awaits further evaluation.

Long and Stone (1985) have observed that inhibition of the background release of EDRF may lead to its buildup within endothelial cells, which results in a large release of EDRF upon restoration of calcium. Therefore, it may be plausible to assume that cumulatively increased calcium in the media causes an initial contraction through its entry into the vascular smooth muscle and a progressive buildup of calcium in the endothelial cell (through an unidentified channel ?) causes a release of EDRF and subsequent relaxation.

Alternatively, EDRF formed elasewhere must be considered. Recent evidence indicates that not only endothelial cells but a variety of cells and tissues including vascular smooth muscle cells (Wood et al, 1990; Busse & Mülsch, 1990; Petitclerc & Marceau, 1991) are able to release nitric oxide. The finding that the magnitude of vasorelaxant attenuation was more pronounced in the preparations treated with L-NAME than was that in the endothelium-denuded is suggestive of an intervention of nitric oxide produced in the cells other than endothelial cells.

On the other hand, stretch of the endothelial membrane, such as might occur with increased intraluminal pressure or high flow rates, causes activation of nonselective cation channels with a significant calcium permeability (Lansman et al, 1987). It may be noteworthy, therefore, to point out that these channels could underlie the observed relaxation in the present study, since the vascular ring was inevitably stretched during the process of isometric recording. An ex-

trapolation of a role for endogenous nitric oxide in the calcium-relaxation observed *in vitro* to an *in vivo* phenomenon must await further clarification.

The prostanoid may be another candidate in eliciting the vasorelaxation. However, the inability of cyclooxygenase inhibitors to prevent the relaxation may exclude prostacyclin as a mediator

Since it has been recognized that changes in osmolality alter cytosolic calcium concentraion (Wang et al, 1992), whether the calcium-induced changes in tension were due to osmolalities has to be answered. However, the same concentration changes induced by neither barium nor sucrose caused a relaxation, in which the final osmolalities were virtually the same. Therefore, the relaxation may not be ascribed to a nonspecific effect(s) such as ionic strength or osmolality.

Finally, the calcium-induced relaxation cannot be attributed to a phenomenon known as "calcium-paradox" (Chapman & Tunstall, 1987), since the contraction-relaxation response could be repeated in the same vascular preparation.

In summary, the present study indicates that the calcium-induced vasorelaxation is, in part, mediated by the release of endogenous nitric oxide.

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