Calcium- and Endothelial-Mediated Vascular Smooth Muscle Relaxation in Rabbit Aorta

HAROLD A. SINGER, B.A., AND MICHAEL J. PEACH, PH.D.

SUMMARY The role of calcium in the relaxations evoked by methacholine and A23187 in intact rabbit aortic rings was investigated. Methacholine (10⁻⁶ to 10⁻⁴ M) and the calcium ionophore A23187 (10⁻⁶ to 10⁻⁴ M) produced dose-dependent relaxations of rings which had been contracted with the α-adrenergic agonist phenylephrine. The ability of a ring to relax in this manner was correlated with the presence of endothelium as judged by transmission and scanning electron microscopy. Purposely disrupting the endothelium led to a loss of the relaxation response. In these rings methacholine caused dose-dependent contractions at concentrations greater than 10⁻⁶ M. Deletion of Ca²⁺ from the incubation medium inhibited maximum methacholine-induced relaxations by 67% and A23187-induced relaxations by 92%. The Ca²⁺-channel blockers verapamil (10 μM) and nifedipine (0.5 μM) inhibited maximum methacholine-induced relaxations by 39% and 45%, respectively. The blockers had no effect on the methacholine ED₅₀ (2.5 × 10⁻⁷ M) for relaxation. Verapamil and nifedipine also inhibited maximum A23187-induced relaxations by 43% and 47% with no effect on the ED₅₀ (6 × 10⁻⁷ M) for relaxation. A structurally dissimilar vasodilator, sodium nitroprusside (10⁻⁴ M), had no effect on the A23187-induced relaxation. These data are consistent with a role of Ca²⁺ in regulating either the production or release of endothelial-derived relaxing factor(s). (Hypertension 4 (suppl II): II-19-II-25, 1982)

KEY WORDS • calcium • methacholine • A23187 • verapamil • nifedipine

The importance of the endothelium in mediating vasodilatory responses is becoming increasingly clear. Prostacyclin, for example, is thought to be derived largely from the endothelium¹, ² and is produced in response to a variety of stimuli. Recently, Furchgott and Zawadzki³ found that several vascular tissues contracted by a variety of agonists relaxed in response to subcontractile concentrations of acetylcholine if the intima of the preparation was undisturbed. Mechanical or enzymatic disruption of the endothelium resulted in a loss of relaxation. Further experiments indicated that the relaxation response could be transferred from an intact rabbit aortic strip to one whose intima had been disrupted. This suggested that chemical factors were mediating the response. Endothelial-dependent acetylcholine-induced relaxations have also been observed in canine femoral⁴ and pulmonary arteries.⁵ The relaxation in response to acetylcholine was antagonized by atropine, indicating that the cholinergic receptor involved was muscarinic.⁵ ⁶ Muscarinic receptors in most tissues appear to be coupled to membrane Ca²⁺-channels.⁷ ⁸ Zawadzki et al.⁷ have shown that the calcium ionophore A23187 can also elicit endothelial-dependent relaxations of rabbit aortic rings. Therefore, we hypothesized that Ca²⁺ was probably an important regulator or stimulus for the production and/or release of the endothelial relaxing factor(s). To test this, we examined the effects of Ca²⁺-deficient buffer and the Ca²⁺-channel blockers verapamil and nifedipine on methacholine- and A23187-induced relaxations in rabbit aortic rings. Our results confirm the importance of endothelium in mediating methacholine- and A23187-induced relaxation in this preparation. Further, we have demonstrated the critical role of Ca²⁺ as a mediator or regulator of endothelial cell production (or release) of the unidentified relaxing substance(s).

Methods

Thoracic aortas were removed from male New Zealand white rabbits (3–4 kg) sacrificed by cervical dislocation. After the aortas were cleaned of superficial adipose and connective tissue, 2–3 mm rings were cut with scissors and mounted in 10 ml water-jacketed organ chambers for tension recording. Care was taken during these steps to avoid unnecessary stretching or contact with the luminal surfaces of the rings. In some preparations the intimal surface was
purposely disrupted by pulling a nylon cord through the lumen of the ring. The rings were maintained at 37°C in Krebs-Ringer buffer of the following composition (millimolar): 111 NaCl, 5 KCl, 1 NaH2PO4, 0.5 MgCl2, 25 NaHCO3, 2.5 CaCl2 and 11.1 dextrose. In preparing buffer deficient in Ca++ (‘0’Ca++), the CaCl2 was omitted and the dextrose increased to 18.6 mM to maintain osmolality. The buffers were gassed continuously in the chamber with 95% O2, 5% CO2. Rings were equilibrated for 1½ hours during which time the buffer was changed at 30-minute intervals. Final resting tension prior to experimentation was 1.5 g. Buffer was changed at 15-minute intervals between experimental periods. Experimental periods were separated by at least 45 minutes. Bath volumes during experimentation were 5 ml. Tension was monitored with Grass (FT03C) force transducers coupled to a Brush 440 recorder.

Phenylephrine (Sigma Chemical Company, St. Louis, Missouri) and acetyl-β-methylcholine chloride (methacholine, Sigma) solutions were prepared daily and diluted into buffer. Verapamil - HCl (Knoll Pharmaceutical Company, Wrippany, New Jersey) and sodium nitroprusside (Sigma) were dissolved in distilled H2O. Nifedipine (Pfizer Pharmaceuticals, New York, New York) was dissolved in dimethysulfoxide (DMSO). A 10^{-5} M stock solution of the calcium ionophore A23187 (Calbiochem-Behring, La Jolla, California) was prepared in DMSO and stored frozen at -20°C. This was thawed daily and an aliquot taken and diluted to appropriate concentrations in distilled water. The cumulative DMSO concentration in the bath did not exceed 0.1%. DMSO at this concentration was found to have no apparent effect on contractile or relaxation responses.

A typical experiment consisted of a control period during which time the rings were contracted by an approximate ED50 concentration (0.5-1.0 × 10^{-9} M) of phenylephrine. After the tension had stabilized (~10 min) the rings were relaxed by the addition of cumulative doses of methacholine or A23187. This was followed, after washing, by a second period during which time drug was introduced or 0’Ca++ buffer substituted. Verapamil and nifedipine were added a minimum of 30 minutes prior to the second contraction-relaxation protocol. When the effect of 0’Ca++ buffer was tested, a brief 5-minute wash in this buffer was followed by the addition of 5 ml fresh 0’Ca++ buffer. At this point the second experimental cycle commenced. Exposure to verapamil or 0’Ca++ buffer reduced but did not eliminate the contractile responsiveness of the rings to phenylephrine. Under these conditions increasing amounts of phenylephrine were titrated to the bath in order to achieve a contraction comparable in magnitude to the control response for that ring. No significant changes were observed in either sensitivity or magnitude of methacholine- or A23187-induced responses over two experimental periods in control rings.

Each experimental group included observations on aortic rings from at least three animals. Paired Student’s t test was used to analyze the effect of Ca++ deficient buffer. All other data were evaluated with unpaired Student’s t test. A p < 0.05 (two-tailed) was considered statistically significant.

Endothelial integrity was judged in several rings by light microscopy, and transmission and scanning electron microscopy. For light and transmission electron microscopy, the rings were fixed for 2 hours by 3% gluteraldehyde in Hank’s balanced salt solution (HBSS), pH 7.2. Rings were postfixed for 1 hour in 1% osmium tetroxide, HBSS pH 7.4. The specimens were then stained en bloc with 5% uranyl acetate for 2 hours, dehydrated in absolute ethanol, and embedded in Polybed (Polysciences). Thick sections (2 μm) were mounted on glass slides for light microscopy. Thin sections (500-700 Å) were mounted on a grid and stained with lead citrate prior to microscopy with a Zeiss model 9A electron microscope. For scanning electron microscopy the rings were fixed for 1.5 hour by 3% gluteraldehyde in 0.1 M cacodylate buffer, pH 7.4. The rings were then postfixed for 1 hour in 1% osmium tetroxide, 0.1 M cacodylate buffer, pH 7.4. After rinsing and careful dehydration in absolute ethanol, the rings were critical-point-dried with CO2 opened with a razor blade to expose their intima, and mounted on a grid. The specimens were sputter coated with gold palladium prior to microscopy with a JOEL 35C scanning electron microscope.

Figure 1 shows several typical scanning electron micrographs. The intact endothelial lining of a control ring fixed immediately upon dissection (fig. 1 a) or a control ring with demonstrated ability to relax in response to methacholine (fig. 1 b) is sharply contrasted by the complete lack of endothelial cells on the luminal surface of a rubbed ring (fig. 1 c). Light and transmission electron microscopy confirmed the lack of endothelium in rubbed rings.

Results

Figure 2 illustrates a representative relaxation in a ring contracted to approximately 3 g with the α-adrenergic agonist phenylephrine. The cholinergic agonist methacholine was added to the bath in cumulative doses (10^{-8} - 10^{-4} M) at the plateau of the phenylephrine-induced contraction. Relaxation occurred with a threshold of approximately 3 × 10^{-8} M. A 50% maximal inhibition in response to 1 × 10^{-8} M methacholine was a typical response in our laboratory (figs. 3 and 4). In rings that were rubbed for the purpose of disrupting endothelium, the relaxation component of the cholinergic response was abolished. Under these conditions, with disrupted endothelium, only a contractile response occurred with concentrations of methacholine greater than 10^{-6} M. In intact rings with higher concentrations of methacholine (usually greater than 10^{-4} M), the contractile component appeared to dominate, and the relaxation was reversed. The calcium ionophore A23187 caused a similar dose-dependent relaxation of intact aortic rings over a range of 10^{-4} - 10^{-8} M (fig. 2). Threshold relaxations have been observed as low with as 6 × 10^{-8}
M A23187. Rubbing the intimal surface abolished the A23187-induced relaxation. Over the concentration range studied, A23187 did not add to the phenylephrine-induced tension in rubbed rings.

To confirm the Ca\(^{++}\) dependence of the A23187- and methacholine-induced relaxations, CaCl\(_2\) was removed from the incubation buffer. A 15-minute exposure to '0'Ca\(^{++}\) buffer (5 minutes during pre-incubation plus 10 minutes during contraction) significantly reduced the magnitude of the methacholine-induced relaxation by 67% when compared to responses obtained in the same rings under control conditions (2.5 mM Ca\(^{++}\); fig. 3). There was no significant change in the sensitivity of the rings to methacholine as judged by comparing ED\(_{50}\)s in the presence and absence of Ca\(^{++}\). It was also apparent from tension recordings that the methacholine relaxations obtained in '0'Ca\(^{++}\) were very transient compared to controls. Under the same conditions of extracellular Ca\(^{++}\) depletion, A23187-induced relaxations were essentially eliminated (92% inhibition) in a second group of rings (fig. 5).

**Figure 1.** Scanning electron micrographs of rabbit aortic ring intima. a. Control ring fixed immediately after dissection, demonstrating an intact covering of endothelial cells. X 480. b. Ring with demonstrated ability to relax in response to methacholine showing generally intact endothelium with some areas of damage. X 1200. c. Ring whose endothelium was purposely disrupted prior to a demonstrated loss of methacholine (relaxation) responsiveness. X 480. d. The same ring at higher magnification shows that this convoluted surface is a fibrous matrix thought to represent the internal elastic lamina. X 2400.
To test the effect of the Ca\(^{++}\)-channel blockers verapamil and nifedipine on the relaxation response, experimental conditions (concentrations of channel blocker and incubation time) were determined which produced little or no inhibition of phenylephrine responsiveness. Nifedipine (0.5 \(\mu\)M) and verapamil (10 \(\mu\)M) added 30 minutes prior to the phenylephrine-induced contraction produced 39% and 45% inhibitions, respectively, in the relaxing efficacy of methacholine, with no change in the sensitivity of the response (ED\(_{50} 2.5 \times 10^{-6}\)M; fig. 4). Verapamil (10 \(\mu\)M) and nifedipine (0.5 \(\mu\)M) caused 43% and 47% inhibitions (\(p < 0.01\)), respectively, in the magnitude of maximum A23187-induced relaxations, with no significant effect on the ED\(_{50} (6 \times 10^{-8}\)M; fig. 6). The nifedipine inhibition was markedly time-dependent.
(Table 1) with incubations beyond 30 minutes producing additional inhibition. As a control, the effect of sodium nitroprusside on A23187-induced relaxations was determined (Table 1). After 10- and 30-minute incubations with \(10^{-7}\) M nitroprusside, significantly greater concentrations of phenylephrine were required to elicit contractions comparable to controls. However, this treatment had no significant effect on either the sensitivity or magnitude of A23187-induced relaxations (Table 1). In another control experiment, 30 minutes of preincubation with \(10^{-6}\) M verapamil had no significant effect upon either the sensitivity (ED\(_{50}\) 9 \(\times\) \(10^{-6}\) M) or magnitude of relaxation (95\% ± 2\%) induced by sodium nitroprusside.

### Table 1. Effect of Nitroprusside (NP) and Time of Exposure to Nifedipine (NFD) on Phenylephrine (PE) Contractions and A23187-Induced Relaxation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No.</th>
<th>Concentration of PE (M)</th>
<th>Developed tension (g)</th>
<th>Maximum (g)</th>
<th>ED(_{50}) (M)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>1.0 ± 0.0 (\times) (10^{-7})</td>
<td>3.03 ± 0.20</td>
<td>2.48 ± 0.17</td>
<td>5 (\times) (10^{-6})</td>
</tr>
<tr>
<td>NFD (0.5 (\mu)M) 30 min</td>
<td>6</td>
<td>0.9 ± 0.1 (\times) (10^{-7})</td>
<td>2.96 ± 0.20</td>
<td>1.86 ± 0.18</td>
<td>6 (\times) (10^{-6})</td>
</tr>
<tr>
<td>NFD (0.5 (\mu)M) 60 min</td>
<td>6</td>
<td>1.7 ± 0.2 (\times) (10^{-7})</td>
<td>2.72 ± 0.20</td>
<td>0.90 ± 0.20</td>
<td>5 (\times) (10^{-6})</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>1.4 ± 0.1 (\times) (10^{-7})</td>
<td>2.52 ± 0.08</td>
<td>1.67 ± 0.07</td>
<td>3 (\times) (10^{-6})</td>
</tr>
<tr>
<td>NP ((10^{-7})) 10 min</td>
<td>7</td>
<td>2.5 ± 0.4 (\times) (10^{-7})</td>
<td>2.62 ± 0.14</td>
<td>1.59 ± 0.22</td>
<td>5 (\times) (10^{-6})</td>
</tr>
<tr>
<td>NP ((10^{-7})) 30 min</td>
<td>5</td>
<td>1.6 ± 0.6 (\times) (10^{-6})</td>
<td>3.52 ± 0.40</td>
<td>1.77 ± 0.21</td>
<td>5 (\times) (10^{-6})</td>
</tr>
</tbody>
</table>

Values are given as means ± SEM.

*Calculated from mean dose-response curve.

\( \ast p < 0.05 \) when compared to control.

\( \ast p < 0.01 \) when compared to control.

**Figure 5.** Effect of Ca\(^{++}\) removal on A23187-induced relaxation in intact aortic rings contracted with phenylephrine. Cumulative dose effect curves are shown. Control rings developed 3.07 (± 0.15) g of tension in response to 2.4 \(\times\) \(10^{-7}\) M of phenylephrine (PE). The '0' Ca\(^{++}\) rings required 5.1 \(\times\) \(10^{-7}\) M PE to develop 2.90 (± 0.16) g. Values given are means (± sem). All values obtained in '0' Ca\(^{++}\) were significantly different from control (\( p < 0.01 \)).

**Figure 6.** Effects of 30-minute preincubation with Ca\(^{++}\)-channel blockers on A23187-induced relaxations in rings contracted by phenylephrine (PE). Cumulative dose-effect curves are shown. Control rings developed 2.84 (± 0.15) g of tension in response to 0.6 \(\times\) \(10^{-3}\) M PE. Verapamil-treated rings required 2.8 \(\times\) \(10^{-3}\) M PE to develop 2.76 (± 0.14) g. The contractile responsiveness of nifedipine-treated rings was not significantly different from controls. Values given are means (± sem). In the presence of Ca\(^{++}\)-channel blockers, all points greater than 3 \(\times\) \(10^{-5}\) M A23187 were significantly different from control (\( p < 0.05 \)).
Discussion

Relaxation of rabbit aortic rings (contracted with the α-adrenergic agonist phenylephrine) by low concentrations of the cholinergic agonist methacholine (10⁻⁶–10⁻⁴ M) as described in this report confirms recent observations by Furchgott and Zawadzki and an earlier report by Jeliffe. A strict dependence of the methacholine-induced relaxation on an intact intima was observed and substantiated by electron microscopy. Similar endothelial-dependent relaxations were observed in response to the antibiotic A23187 (10⁻⁵–10⁻⁴ M). A23187 is a well-documented ionophore with two effects on vascular smooth muscle: 1) extracellular depolarization of the smooth muscle cell; 2) entry of Ca²⁺ into the smooth muscle cell. Removal of Ca²⁺ from the incubation buffer virtually eliminates the A23187-induced relaxation and severely depresses the methacholine-induced relaxation, suggesting similar mechanisms of action for the two stimuli. Although not shown, recordings clearly indicate very transient methacholine relaxations in 0 Ca²⁺ buffer quite unlike those observed in normal Ca²⁺. These observations suggest that methacholine may be able to utilize pools of Ca²⁺ inaccessible to the ionophore A23187.

Because of difficulties in interpreting the precise cellular events altered by Ca²⁺ depletion, we sought to determine the effect of Ca²⁺-channel blockers on the endothelial-mediated relaxation response in buffer complete with Ca²⁺. Verapamil and nifedipine have both been shown to block voltage-sensitive Ca²⁺ channels in vascular tissues. Verapamil and cinnarizine, another Ca²⁺-channel blocker, have also been shown to have little effect on norepinephrine-induced contraction in rabbit thoracic aorta. Explanations for this have centered on the ability of norepinephrine to utilize intracellular pools of Ca²⁺, unaffected by the Ca²⁺-channel blockers, as sources of activator Ca²⁺ for contraction.

We reasoned that these Ca²⁺-channel blockers held potential as probes for examining the Ca²⁺-dependence of the endothelial vasodilator pathway in rabbit aorta with little interference from vascular smooth muscle effects. Methacholine-induced relaxations were found to be significantly inhibited in the presence of either nifedipine (0.5 μM, −39%) or verapamil (10 μM, −45%). This is consistent with activation of endothelial Ca²⁺-channels by methacholine. The resistance of at least half of the methacholine response to these blockers suggests that this agonist may utilize multiple pools of Ca²⁺. A23187-induced relaxations were similarly inhibited by nifedipine (−47%) and verapamil (−43%), suggesting a mechanism of action for the ionophore similar to methacholine. These results would be unexpected if A23187 was acting simply as an ionophore translocating Ca²⁺ independently of endogenous membrane Ca²⁺-channels. There are several potential explanations for these observations. Perhaps the simplest is that direct interaction of the Ca²⁺-channel blockers with A23187. Such a mechanism has been proposed to explain the inhibitory effect of verapamil on A23187-induced Ca²⁺ uptake in isolated hepatocytes. A direct interaction of verapamil with A23187 and subsequent inhibition of ionophore-mediated Ca²⁺ translocation from an aqueous phase into organic solvents has been demonstrated. A more complex explanation consistent with Ca²⁺-channel blocking activity is that increased intracellular Ca²⁺ induced by A23187 may trigger endothelial-cell depolarization with subsequent Ca²⁺-channel activation. This mechanism has been proposed to explain A23187-induced depolarizations of pancreatic acinar cells which were Na⁺- as well as Ca²⁺-dependent. Alternatively, prolonged blockade of Ca²⁺-channels may promote a redistribution of intracellular Ca²⁺ out of pools normally utilized by A23187.

While nifedipine (0.5 μM) had no apparent effect on the sensitivity of rings to the contractile agonist phenylephrine, verapamil (10 μM), and calcium-deficient buffer significantly depressed responsiveness. It is possible that, when compared to controls, the generally higher level of α-receptor activation required to elicit comparable contractions under these conditions somehow interfered with or antagonized the relaxing mechanism of the endothelial factor(s).

To rule this out, sodium nitroprusside, a vasodilator structurally and mechanistically dissimilar to Ca²⁺-channel blockers, was administered to reduce responsiveness to phenylephrine. Ten times more phenylephrine was required to achieve control magnitude contractions after a 30-minute incubation with 10⁻⁷M nitroprusside yet the relaxation in response to A23187 was unaltered. As a further control, we determined that the intrinsic ability of a contracted ring to relax in response to nitroprusside was not depressed by the prior addition of 10 μM verapamil. From these data it appears that the Ca²⁺-channel blockers are exerting relatively specific inhibitory effects upon the endothelial-dependent relaxations. However, we cannot distinguish between effects on synthesis, release, or sensitivity to the relaxing factor(s).

Three lines of evidence directly support a role for Ca²⁺ in mediating or regulating the endothelial dependent methacholine-induced relaxation: 1) extracellular Ca²⁺ depletion inhibits the methacholine-induced relaxation; 2) the calcium ionophore A23187 induces similar Ca²⁺-dependent relaxations; 3) the Ca²⁺-channel blockers nifedipine and verapamil can inhibit both methacholine- and A23187-induced relaxations.

The Ca²⁺ dependence of the methacholine response is consistent with the Ca²⁺ dependence of other muscarinic responses such as contraction in smooth muscle or secretion in exocrine cells. A potential mechanism of action for methacholine as a relaxing agonist in intact rabbit aorta involves the activation of phospholipases with subsequent release of arachidonic acid. This mechanism would also be consistent with the observed Ca²⁺ dependence of the relaxation response. Phospholipases, especially phospholipase A₂, appear in many tissues to be activated in a Ca²⁺-dependent manner by specific hormonal stimuli or the Ca²⁺ ionophore A23187.
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Calcium- and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta.
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