Antagonistic Modulatory Roles of Magnesium and Calcium on Release of Endothelium-Derived Relaxing Factor and Smooth Muscle Tone

Michele E. Gold, Georgette M. Buga, Keith S. Wood, Russell E. Byrns, Gautam Chaudhuri, and Louis J. Ignarro

The objective of this study was to elucidate the mechanisms associated with the reciprocal relation between magnesium and calcium on vascular smooth muscle tone in bovine pulmonary artery and vein. Rapid removal of magnesium from Krebs-bicarbonate medium used to bathe isolated rings of precontracted artery or vein caused transient endothelium- and calcium-dependent relaxation and cyclic GMP accumulation. Both responses were antagonized by oxyhemoglobin, methylene blue, or superoxide anion and were enhanced by superoxide dismutase. The transient relaxation was followed by sustained endothelium-independent contraction. Endothelium-denuded vascular rings contracted in response to extracellular magnesium depletion without alteration in cyclic GMP levels. The data suggest that vascular endothelium-derived nitric oxide is responsible for the calcium-dependent relaxation elicited by extracellular magnesium depletion. Indeed, in bioassay cascade studies, magnesium removal from the medium used to perfuse intact artery or vein enhanced the formation and/or release of an endothelium-derived relaxing factor by calcium-dependent mechanisms. In the absence of both extracellular magnesium and calcium, calcium readdition caused transient endothelium-dependent relaxation and cyclic GMP accumulation, and both responses were abolished by oxyhemoglobin or methylene blue. In the presence of magnesium, however, readdition of calcium to calcium-depleted medium caused only contractile responses. Addition of magnesium to calcium-containing medium consistently caused endothelium- and cyclic GMP–independent relaxation that was not altered by oxyhemoglobin or methylene blue. Thus, magnesium and calcium elicit reciprocal or mutually antagonistic effects at the levels of both endothelium-derived relaxing factor formation and/or release and smooth muscle contraction. This relation may be of physiological importance, and the possibility that a reduction in circulating magnesium levels could lead to calcium-mediated vasospasm may be of pathophysiological concern. (Circulation Research 1990;66:355–366)

Since the initial discovery in 1980 that acetylcholine causes endothelium-dependent arterial relaxation through the release of an endothelium-derived relaxing factor (EDRF),1 much has been learned about the factors influencing the formation and/or release of EDRF.2–4 Other than prostacyclin, more than one EDRF is likely to exist, but only one has been identified. An EDRF released from perfused cultured aortic endothelial cells5 and from perfused intact artery and vein6,7 has been identified as nitric oxide or a closely related labile nitroso species that spontaneously liberates nitric oxide on release from endothelial cells. This endothelium-derived nitric oxide (EDNO) appears to account for the biological actions of EDRF in vascular smooth muscle and platelets,5–12 and such actions are indistinguishable from those first described for authentic nitric oxide almost a decade ago.13–16

Extracellular calcium appears to be essential for endothelium-dependent vascular smooth muscle relaxation.17–20 and other studies have shown that endothelium-dependent relaxation is dependent on
the intracellular concentration of calcium in the endothelial cells.\textsuperscript{31,32} The influx of extracellular calcium may be one mechanism that couples the interaction of endothelium-dependent vasodilators at endothelial cell surface receptors to the synthesis and/or release of EDRF, although some controversy exists over the precise mechanisms involved.\textsuperscript{3,17–20,22–26} Extracellular magnesium has been found to inhibit calcium influx at the vascular smooth muscle membrane\textsuperscript{27–29} and at the myocardial sarcolemmal membrane\textsuperscript{30} and has been suggested to interfere with calcium release from intracellularly bound sites in vascular smooth muscle.\textsuperscript{31} Such a reciprocal or antagonistic relation between magnesium and calcium has been recently described also with respect to endothelium-dependent vascular smooth muscle relaxation, in which extracellular magnesium depletion caused endothelium- and calcium-dependent arterial relaxation.\textsuperscript{32} The objective of the present study was to address the latter observations and elucidate the mechanism of the apparently mutual antagonistic relation between extracellular magnesium and calcium on endothelium-dependent relaxation in bovine intrapulmonary artery and vein. To this end, the influence of extracellular magnesium and calcium on the formation and/or release of EDRF, endothelium-dependent and -independent relaxant responses, and cyclic GMP formation in vascular smooth muscle was examined.

\textbf{Materials and Methods}

\textit{Preparation of Rings of Bovine Intrapulmonary Artery and Vein}

Bovine lungs were obtained from cows 5 years of age or older and transported to the laboratory in iced Krebs-bicarbonate solution as described.\textsuperscript{33} The second intrapulmonary arterial branch and underlying venous branch extending into the larger lobe were rapidly excised, gently cleaned of parenchyma, fat, and adhering connective tissue, and placed in cold preoxygenated Krebs-bicarbonate solution. Segments with outside diameters of 4–6 mm (artery) and 6–8 mm (vein) were isolated and sliced into rings (4 mm wide) with a specially designed microtome.\textsuperscript{34} Rings were prepared with an intact or functional endothelium as assessed by 80–100% relaxation to 0.1–1 \( \mu \)M acetylcholine in artery and 10 nM bradykinin in vein. Endothelial cells were removed from some arterial or venous rings and are referred to in the text as either endothelium-denuded or rubbed. Endothelium-denuded arterial or venous rings were prepared by gently evertting the rings (intimal side out) and rubbing the entire surface with moistened filter paper for 30 seconds; rings were then returned to their normal position (intimal side in). These endothelium-denuded rings contracted in response to 1 \( \mu \)M acetylcholine (artery) or 10 nM bradykinin (vein).

\textit{Mounting Rings and Recording of Muscle Tension}

Arterial and venous rings were mounted on nichrome wires in jacketed, 25-ml capacity, drop-
from the perfusion medium, the superfusion medium was adjusted to twice the magnesium concentration to correct for the dilution caused by mixing with the magnesium-free perfusate. Similar procedures were followed when the calcium concentration in the perfusion medium was altered.

**Determination of Cyclic Nucleotide Levels**

Cyclic GMP levels were measured in arterial and venous rings that had been equilibrated under tension and depolarized with KCl. Tone was monitored until the time of freeze-clamping. The rapid drop-away bath chambers were lowered, and rings were quickly frozen between brass clamps precooled in liquid nitrogen. Each frozen ring was homogenized in 1 ml of 6% trichloroacetic acid (ground-glass tissue grinder). After centrifugation, the supernatant was extracted with diethyl ether to remove the acid, and aliquots of the aqueous phase were lyophilized to dryness, reconstituted in buffer, and analyzed by radioimmunoassay. These procedures were described previously.14,34 None of the test agents added to the bath chambers interfered directly with antigen-antibody binding in the radioimmunoassay procedures. Recoveries of standard amounts of added cyclic nucleotides were determined periodically, and the values ranged from 92% to 104%. Therefore, no corrections for sample recoveries were made.

**Chemicals and Solutions**

Acetylcholine chloride, bradykinin triacetate, A23187, phenylephrine hydrochloride, indomethacin, hemoglobin, sodium dithionite, methylene blue, pyrogallol, and superoxide dismutase (bovine liver) were obtained from Sigma Chemical, St. Louis, Missouri. U46619 ([153I]-hydroxy-11α,9α-epoxymethano]prostaz5Z,13E-dienoic acid) was provided by The Upjohn Co., Kalamazoo, Michigan, and was dissolved in absolute ethanol at a concentration of 10 mg/ml. Dilutions were prepared in cold distilled water to a final concentration of 0.1 mM and stored frozen. S-Nitroso-N-acetylpenicillamine was synthesized and used as described previously.36 Glyceryl trinitrate (10% wt/wt triturated mixture in lactose) was a gift from ICI.
### Table 1. Influence of Alterations in Magnesium and Calcium Concentrations on Endothelium-Dependent and -Independent Responses

<table>
<thead>
<tr>
<th>Test condition</th>
<th>n</th>
<th>Endothelium</th>
<th>Percent relaxation</th>
<th>Percent contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>36</td>
<td>+</td>
<td>37±8</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>–</td>
<td>...</td>
<td>18±4</td>
</tr>
<tr>
<td>2. 1.2 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>18</td>
<td>+</td>
<td>36±6</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>–</td>
<td>38±8</td>
<td>...</td>
</tr>
<tr>
<td>3. Δ-Mg&lt;sup&gt;2+&lt;/sup&gt; (vein)</td>
<td>18</td>
<td>+</td>
<td>26±3</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>–</td>
<td>...</td>
<td>12±2</td>
</tr>
<tr>
<td>4. 1.2 mM Mg&lt;sup&gt;2+&lt;/sup&gt; (vein)</td>
<td>12</td>
<td>+</td>
<td>31±4</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>–</td>
<td>30±3</td>
<td>...</td>
</tr>
<tr>
<td>5. 1 μM HbO&lt;sub&gt;2&lt;/sub&gt;, Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>18</td>
<td>+</td>
<td>...</td>
<td>14±2*</td>
</tr>
<tr>
<td>6. 10 μM MB, Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>18</td>
<td>+</td>
<td>...</td>
<td>12±1*</td>
</tr>
<tr>
<td>7. 100 units/ml SOD+Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>12</td>
<td>+</td>
<td>100±0</td>
<td>...</td>
</tr>
<tr>
<td>8. 100 units/ml SOD+Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>12</td>
<td>–</td>
<td>...</td>
<td>14±2</td>
</tr>
<tr>
<td>9. 0.1 mM pyrogallol+Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>12</td>
<td>+</td>
<td>...</td>
<td>20±3*</td>
</tr>
<tr>
<td>10. 1 μM HbO&lt;sub&gt;2&lt;/sub&gt;, Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;, 1.2 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>18</td>
<td>+</td>
<td>33±5</td>
<td>...</td>
</tr>
<tr>
<td>11. 10 μM MB, Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;, 1.2 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>12</td>
<td>+</td>
<td>30±4</td>
<td>...</td>
</tr>
<tr>
<td>12. −Ca&lt;sup&gt;2+&lt;/sup&gt;, Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>24</td>
<td>+</td>
<td>...</td>
<td>13±2*</td>
</tr>
<tr>
<td>13. −Ca&lt;sup&gt;2+&lt;/sup&gt;, Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;, 1.5 mM Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>24</td>
<td>+</td>
<td>55±8</td>
<td>...</td>
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<tr>
<td>14. 1 μM HbO&lt;sub&gt;2&lt;/sub&gt;, −Ca&lt;sup&gt;2+&lt;/sup&gt;, Δ-Mg&lt;sup&gt;2+&lt;/sup&gt;, 1.5 mM Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>12</td>
<td>+</td>
<td>...</td>
<td>7±1†</td>
</tr>
<tr>
<td>15. Acetylcholine</td>
<td>12</td>
<td>+</td>
<td>11±2</td>
<td>...</td>
</tr>
<tr>
<td>0.01 μM</td>
<td>12</td>
<td>+</td>
<td>44±6</td>
<td>...</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>12</td>
<td>+</td>
<td>89±11</td>
<td>...</td>
</tr>
<tr>
<td>1 μM</td>
<td>12</td>
<td>+</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>16. −Ca&lt;sup&gt;2+&lt;/sup&gt;, acetylcholine</td>
<td>12</td>
<td>+</td>
<td>0±0‡</td>
<td>...</td>
</tr>
<tr>
<td>0.01 μM</td>
<td>12</td>
<td>+</td>
<td>9±1‡</td>
<td>...</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>12</td>
<td>+</td>
<td>20±2‡</td>
<td>...</td>
</tr>
<tr>
<td>1 μM</td>
<td>12</td>
<td>+</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>17. −Ca&lt;sup&gt;2+&lt;/sup&gt;, −Mg&lt;sup&gt;2+&lt;/sup&gt;, 1.5 mM Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>24</td>
<td>+</td>
<td>52±8</td>
<td>...</td>
</tr>
<tr>
<td>18. 1 μM HbO&lt;sub&gt;2&lt;/sub&gt;, −Ca&lt;sup&gt;2+&lt;/sup&gt;, −Mg&lt;sup&gt;2+&lt;/sup&gt;, 1.5 mM Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>12</td>
<td>+</td>
<td>...</td>
<td>66±9§</td>
</tr>
<tr>
<td>19. −Mg&lt;sup&gt;2+&lt;/sup&gt;, acetylcholine</td>
<td>12</td>
<td>+</td>
<td>14±2</td>
<td>...</td>
</tr>
<tr>
<td>0.01 μM</td>
<td>12</td>
<td>+</td>
<td>43±6</td>
<td>...</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>12</td>
<td>+</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>1 μM</td>
<td>12</td>
<td>+</td>
<td>92±12</td>
<td>...</td>
</tr>
</tbody>
</table>

Americas, Wilmington, Delaware. Solutions of hydroscopic acetylcholine were prepared in distilled water, separated into aliquot portions, and stored frozen. Bradykinin, pyrogallol, A23187, and superoxide dismutase were prepared fresh in distilled water just before use. Oxyhemoglobin was prepared from hemoglobin by reduction with sodium dithionite in oxygenated Krebs-bicarbonate solution at 4°C, essentially as described.7 Krebs-bicarbonate solution consisted of (mM) NaCl 118, KCl 4.7, CaCl<sub>2</sub> 1.5, NaHCO<sub>3</sub> 25, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, and glucose 11. Depolarizing KCl solution had a composition similar to Krebs-bicarbonate solution except the sodium chloride was replaced with an equimolar concentration of KCl.

**Calculations and Statistical Analysis**

Relaxation and contraction were measured as the decrease and increase, respectively, in tension relative to the tension elicited by precontracting arterial or venous smooth muscle with phenylephrine or U46619 as indicated. Values in Figures 7–10 are expressed as the mean±SEM and represent unpaired data. Comparisons were made by Duncan’s multiple range test<sup>19</sup> for comparisons with a common control (Figures 8–10) or by Student’s t test for unpaired values for all other comparisons where indicated. The level of statistically significant difference was p<0.05. Figures 1–4 illustrate typical tracings from representative experiments. A summary of all of the pertinent data related to the experiments illustrated in Figures 1–4 represented as the mean±SEM, together with a statistical evaluation of important differences, is presented in Table 1.

**Results**

**Characteristics of Relaxation of Artery and Vein Elicited by Removal and Addition of Extracellular Magnesium**

Rapid removal of magnesium from the extracellular medium produced a rapid and transient
TABLE 1. Continued

<table>
<thead>
<tr>
<th>Test condition</th>
<th>n</th>
<th>Endothelium</th>
<th>Percent relaxation</th>
<th>Percent contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>20. 1 μM HbO2, -Mg2+, acetylcholine 1 μM</td>
<td>12</td>
<td>+</td>
<td>14±1</td>
<td>...</td>
</tr>
<tr>
<td>21. -Ca2+, -Mg2+, 1.5 mM Ca2+</td>
<td>24</td>
<td>-</td>
<td>82±12</td>
<td>...</td>
</tr>
<tr>
<td>22. SNAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 μM</td>
<td>12</td>
<td>-</td>
<td>22±3</td>
<td>...</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>12</td>
<td>-</td>
<td>59±8</td>
<td>...</td>
</tr>
<tr>
<td>1 μM</td>
<td>12</td>
<td>-</td>
<td>99±8</td>
<td>...</td>
</tr>
<tr>
<td>23. -Mg2+, SNAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 μM</td>
<td>12</td>
<td>-</td>
<td>18±2</td>
<td>...</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>12</td>
<td>-</td>
<td>54±7</td>
<td>...</td>
</tr>
<tr>
<td>1 μM</td>
<td>12</td>
<td>-</td>
<td>97±6</td>
<td>...</td>
</tr>
<tr>
<td>24. 1 μM HbO2, -Mg2+, SNAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 μM</td>
<td>12</td>
<td>-</td>
<td>2±1</td>
<td>...</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>12</td>
<td>-</td>
<td>14±2</td>
<td>...</td>
</tr>
<tr>
<td>1 μM</td>
<td>12</td>
<td>-</td>
<td>35±4</td>
<td>...</td>
</tr>
</tbody>
</table>

Unrubbed (+) and endothelium-denuded (−) rings of bovine pulmonary artery or vein were mounted and equilibrated as described in the text. Rings were precontracted (65–75% of maximal) by addition of phenylephrine. Data represent the mean±SEM, and n signifies the number of separate vascular rings tested. Percent relaxation and contraction, respectively, signify percentage decrease and increase in phenylephrine-induced tone. Test conditions were as follows: 1, rapid replacement of Krebs’ bathing medium with Mg2+-free medium at time of peak contractile response; 2, added after stabilization of tone in response to -Mg2+; 3, same as 1; 4, same as 2; 5, oxyhemoglobin (HbO2) added 10 minutes before precontraction, and -Mg2+ added at peak contraction; 6, similar to 5 (MB, methylene blue); 7 and 8, superoxide dismutase (SOD) included in Mg2+-free medium at -Mg2+; 9, similar to 7; 10, HbO2 added 10 minutes before contraction, -Mg2+ added at peak contraction, and Mg2+ added after stabilization of tone; 11, similar to 10; 12, replacement of Krebs’ bathing medium with Ca2+-free medium 10 minutes before contraction, and -Mg2+ added at peak contraction; 13, same as 12 except Ca2+ added back after stabilization of tone; 14, same as 13 except HbO2 added 10 minutes before contraction; 15, cumulative addition of acetylcholine starting at peak contraction; 16, same as 15 except Krebs’ bathing medium replaced with Ca2+-free medium 10 minutes before contraction; 17, replacement of Krebs’ bathing medium with Ca2+- and Mg2+-free medium 10 minutes before contraction and Ca2+ added back at peak contraction; 18, same as 17 except HbO2 added 10 minutes before contraction; 19, replacement of Krebs’ bathing medium with Mg2+-free medium 10 minutes before contraction and cumulative addition of acetylcholine starting at peak contraction; 20, similar to 19 except HbO2 added 10 minutes before contraction; 21, same as 17; 22, similar to 15 (SNAP, S-nitroso-N-acetylpenicillamine); 23, similar to 19; 24, similar to 20.

- *Significantly different (p<0.05) from values corresponding to test condition 1.
- †Significantly different (p<0.05) from values corresponding to test condition 13.
- ‡Significantly different (p<0.05) from values corresponding to test condition 15.
- §§Significantly different (p<0.05) from values corresponding to test condition 17.
- ††Significantly different (p<0.05) from values corresponding to test condition 19 (1 μM).
- *†Significantly different (p<0.05) from values corresponding to test condition 22.

Endothelium-dependent relaxation of artery (Figure 1) and vein (Figure 2) followed by return of tone that was often greater than precontractile tension (Table 1). Endothelium-denuded arterial and venous rings failed to relax but did contract in response to magnesium removal. Acetylcholine and bradykinin were used to further assess the endothelium-dependent relaxation of intrapulmonary artery and vein, respectively, when exposed to magnesium-free medium. Removal of magnesium from the extracellular medium had no appreciable inhibitory effect on the relaxation caused by these endothelium-dependent relaxants (Table 1). In endothelium-denuded rings exposed to magnesium-free medium, relaxation caused by glyceryl trinitrate was intact with maximal relaxation occurring at 10 nM. Readoption of magnesium to the magnesium-depleted medium resulted in endothelium-independent relaxant responses. To further characterize the endothelium-dependent relaxation produced by magnesium removal and the endothelium-independent relaxation produced by magnesium addition, the effects of oxyhemoglobin, methylene blue, superoxide dismutase, and pyrogallol were assessed. Pretreatment of arterial rings with 1 μM oxyhemoglobin or 10 μM methylene blue abolished the endothelium-dependent relaxation produced by the removal of extracellular magnesium but had no effect on the relaxation produced by the readdiction of magnesium (Table 1). Superoxide dismutase markedly potentiated, whereas pyrogallol antagonized, the endothelium-dependent relaxation produced by removal of extracellular magnesium (Table 1). The effects of the above test agents on venous rings were qualitatively similar to those on arterial rings (data not shown).

**Interaction of Magnesium and Calcium in Endothelium-Dependent Relaxation**

The endothelium-dependent relaxation produced by removal of extracellular magnesium was also calcium-dependent since relaxation did not occur when calcium was removed from the bathing medium (Figure 3). Readddition of calcium resulted in return of the endothelium-dependent relaxant responses, and these were inhibited by 1 μM oxyhemoglobin (Figures 3 and 4, Table 1). Figure 4 shows the...
reciprocal relation between magnesium and calcium and the requirement of calcium for endothelium-dependent relaxant responses to extracellular magnesium removal and to acetylcholine. Adding calcium to arterial rings equilibrated in medium without magnesium or calcium caused a rapid endothelium-dependent relaxation that did not occur in endothelium-denuded rings. This relaxation was inhibited by 1 μM oxyhemoglobin. In the presence of oxyhemoglobin, the subsequent addition of calcium caused a contractile response. Endothelium-denuded arterial rings contracted in response to calcium, whereas S-nitroso-N-acyetylpenicillamine, an unstable nitrovasodilator that generates nitric oxide, caused relaxation that was antagonized by oxyhemoglobin (Figure 4, Table 1).

**Influence of Magnesium and Calcium on Formation/Release of EDRF**

The bioassay cascade technique was used to determine the effects of magnesium (Figure 5) and calcium (Figure 6) on the formation and/or release of arterial or venous EDRF. EDRF released from endothelium-intact bovine pulmonary artery that was perfused with acetylcholine or A23187 was enhanced when magnesium was removed from the perfusion medium. Bovine pulmonary vein was perfused with bradykinin or A23187, and EDRF release was similarly enhanced when magnesium was removed from the perfusion medium. The magnesium concentration of the superfusion medium was increased when magnesium-free perfusion medium was used to maintain the fluid bathing the cascade of vascular strips at a constant magnesium concentration of 1.2 mM. Glycerol trinitrate, which was superfused over the vascular strips, caused relaxation that was not affected by the removal of magnesium from the perfusion medium in the presence of superfused magnesium. The requirement of calcium for EDRF formation and/or release was further substantiated by bioassay because removal of calcium from the
perfusion medium nearly abolished relaxation of vascular strips of artery (Figure 6) or vein (not shown) when endothelium-intact vessels were perfused with acetylcholine, bradykinin, or A23187. Calcium (1.5 mM) was always present in the superfusion fluid bathing the strips. Relaxation of the vascular strips was restored by readdition of calcium to the perfusion medium containing bradykinin (Figure 6). The magnitude of the relaxation was dependent on the concentration of calcium added. Oxyhemoglobin abolished the relaxant responses produced by the removal of magnesium from (Figure 5) or the addition of calcium to (Figure 6) the perfusion medium.

Mediation by Cyclic GMP of Endothelium-Dependent Relaxation Elicited by Magnesium Removal and Calcium Addition

Extracellular magnesium removal produced a rapidly developing, endothelium-dependent fourfold increase in arterial cyclic GMP levels that was associated with relaxation (Figure 7). Removal of extracellular calcium in the presence of magnesium caused smooth muscle relaxation, which was greater in rubbed than intact arterial rings, without appre-
ciably altering resting cyclic GMP levels. The endothelium-dependent increase in cyclic GMP levels and relaxation of arterial rings in response to extracellular magnesium depletion were abolished by oxyhemoglobin or methylene blue (Figure 8). Both inhibitors decreased cyclic GMP levels below control resting levels and reversed relaxation to contractile responses.

Equilibration of arterial rings in magnesium-free medium for 15 minutes caused a twofold increase in cyclic GMP levels, and readdition of magnesium to the precontracted rings caused cyclic GMP–independent relaxant responses (Figure 9). Neither oxyhemoglobin nor methylene blue altered such relaxations although arterial cyclic GMP levels were lowered significantly. Thus, magnesium-elicited vascular smooth muscle relaxation occurred independent of EDRF (Figures 1 and 2) or cyclic GMP (Figure 9).

In contrast, vascular smooth muscle relaxation produced by readdition of calcium to magnesium-depleted extracellular medium was EDRF dependent (Figures 3 and 4) and associated with elevated cyclic GMP levels (Figure 10). The relaxation and increase in cyclic GMP levels were abolished by oxyhemoglobin or methylene blue (Figure 10).

**Discussion**

One purpose of this study was to develop a better understanding of the influence of magnesium and calcium on that component of endothelium-dependent vascular smooth muscle relaxation attributed primarily to EDNO. Nitric oxide or a labile nitroso compound was identified both chemically and pharmacologically as one substance that can account for the biological actions of EDRF released from intact artery and vein as well as from freshly harvested and cultured aortic endothelial cells. In the present study, acetylcholine and bradykinin were used as endothelium-dependent relaxants of isolated precontracted rings of bovine intrapulmonary artery and vein, respectively, because such effects are known to be mediated by EDNO and not by cyclooxygenase...
products of arachidonic acid metabolism. In the bioassay cascade procedure, acetylcholine or A23187 was used to elicit release of EDRF from perfused artery and bradykinin, or A23187 was used in perfused vein, but indomethacin was always present in the perfusion and superfusion media to exclude the relaxant actions of prostacyclin or other eicosanoids. Oxyhemoglobin abolished or nearly abolished the relaxant effects of EDRF released from both perfused artery and vein. These observations are consistent with previous findings that this relaxant action is attributed to a single EDRF identified chemically and pharmacologically as nitric oxide or a chemically related unstable nitroso compound.

The major findings in this study are that depletion of extracellular magnesium causes transient endothelium-dependent and calcium-dependent relaxation not only of artery but also vein, that relaxation is accompanied by endothelium-dependent cyclic GMP accumulation, and that such effects are attributed to the enhanced formation and/or release of an EDRF that is most likely nitric oxide (EDNO). Extracellular magnesium depletion–elicited relaxation and cyclic GMP formation are antagonized by oxyhemoglobin, methylene blue, and superoxide anion generated from pyrogallol, whereas such responses are enhanced by superoxide dismutase. When the bioassay cascade technique is used, the depletion of magnesium from the perfusion medium causes a marked enhancement of formation and/or release of EDRF. Similar observations were made when simultaneous removal of magnesium from both perfusion and superfusion media was performed. Thus, magnesium depletion does not appear to alter the responsiveness of smooth muscle to EDRF. The approximate estimated half-life of EDRF is 3–5 seconds, and relaxant responses in the superfused strips are antagonized by oxyhemoglobin. Based on earlier studies from this and other laboratories where similar experimental approaches were taken, these observations provide evidence that EDNO is responsible for the vascular actions of extracellular magnesium depletion.
The above observations on extracellular magnesium depletion in the presence of calcium are mimicked by the addition of calcium to bathing medium or perfusion medium previously depleted of both calcium and magnesium. Thus, calcium causes endothelium-dependent arterial and venous relaxation and cyclic GMP accumulation, and such vascular responses are attributed to EDNO because they are antagonized by oxyhemoglobin or methylene blue and because the EDRF from perfused artery and vein possesses all of the properties of nitric oxide. These data suggest that extracellular magnesium and calcium elicit mutually antagonistic or reciprocal actions at the level of the formation and/or release of EDNO. Present bioassay procedures as conducted, however, are incapable of distinguishing between formation and release of EDNO.

Another clear expression of the opposing biological actions of magnesium and calcium derives from experiments conducted with endothelium-denuded rings of artery or vein. Under these conditions extracellular magnesium depletion does not cause relaxation but, rather, causes sustained contractions. Similarly, addition of calcium to media deprived of both calcium and magnesium causes only contractile responses. It is notable that even in the presence of a functional endothelium the characteristic transient relaxation caused by extracellular magnesium depletion is quickly followed by a sustained contractile response. The latter contractile response appears to be attributed to the unopposed smooth muscle actions of calcium because addition of excess calcium (greater than 1.5 mM) to endothelium-intact rings in the presence of extracellular magnesium causes only contractile responses. Particularly intriguing are the observations that excess extracellular magnesium (>1.2 mM) elicits marked endothelium-independent and cyclic GMP–independent relaxant responses that are unaltered by oxyhemoglobin, methylene blue, or indomethacin. One plausible explanation for these findings is simply that excess magnesium competes with and overrides the smooth muscle contractile effects of calcium. The precise mechanism of this cation interaction or whether it occurs at extracellular surface ion channels or intracellularly is presently unknown.

Removal of extracellular magnesium caused a marked endothelium-dependent increase in tissue cyclic GMP levels within several minutes, which gradually declined over a 15-minute period but remained at levels that were about twofold higher than control basal levels. This was undoubtedly due to increased EDRF generation or release. Readdition of magnesium did not cause a decrease (within 5 minutes) to cyclic GMP levels to control values, as one might have expected, due to an antagonistic action of magnesium on endothelial cell calcium. It appears that the sustained small elevation in cyclic GMP levels remaining after magnesium readdition is due to sustained EDNO action because oxyhemoglobin or methylene blue rapidly lowered cyclic GMP levels. The reason for this sustained, oxyhemoglobin-sensitive, small elevation in cyclic GMP levels is unknown.

Magnesium and calcium appear to elicit opposing or antagonistic actions on at least two different levels of cellular function. One site is at the endothelial cell on the formation and/or release of EDRF, and the other site is at the smooth muscle cell on processes that lead to calcium-mediated contraction. Because calcium is obligatory for both smooth muscle contraction and EDRF formation and/or release and because magnesium opposes the actions of calcium at both sites, vascular smooth muscle responsiveness to alterations in the extracellular concentration of magnesium and calcium reflects the algebraic sum of the responses. Thus, the magnitude of the relaxation in response to sudden extracellular magnesium depletion or addition of excess extracellular magnesium or removal of extracellular calcium in the presence of magnesium varies from one experiment to another. The dual influence of calcium on endothelial and smooth muscle cells is borne out also by the observations that the removal of extracellular calcium in the presence of magnesium causes a greater magnitude of relaxation in rubbed than endothelial-intact arterial rings. That is, in intact rings calcium both stimulates EDRF formation and/or release and causes contractions, whereas in rubbed rings calcium causes only contraction. Thus, the sudden removal of calcium in the presence of magnesium would be expected to cause greater relaxation of the rubbed arterial ring.

In calcium-free extracellular medium acetylcholine elicited small but significant endothelium-dependent relaxant responses, whereas magnesium withdrawal did not. The reason for this difference is unknown but may be attributed to the release of intracellular calcium by acetylcholine in the endothelial cells. There is evidence that endothelium-dependent relaxation elicited by acetylcholine, bradykinin, and other agonists is dependent also on the presence of both extracellular and intracellular calcium.3,17,22

Some of the observations made in the present study are consistent with those reported previously in which extracellular magnesium depletion caused endothelium- and calcium-dependent (EDRF-mediated) relaxation of isolated canine coronary artery but caused contraction of endothelium-denuded artery.32 One difference between the above study and the present one is that we were unable to observe contractile effects of magnesium added back to magnesium-deficient bathing media in which arterial preparations had just relaxed in response to the removal of magnesium. Instead, we always observed relaxant responses to added magnesium under any conditions, and such responses occurred independent of endothelium or the extracellular concentration of magnesium. Furthermore, in our experiments the relaxations elicited by extracellular magnesium depletion were transient and were followed by sustained contractions, whereas in the former study52.
sustained relaxations were produced. The reasons for these differences are unknown but may be attributed to differences in responsiveness of coronary versus intrapulmonary vessels.

The observations in the present study differ somewhat from those of another study on coronary artery in that endothelium-dependent relaxation of bovine intrapulmonary artery and vein was not dependent on the presence of extracellular magnesium, whereas magnesium appeared to be required for endothelium-dependent canine coronary arterial relaxation. Again, the reasons for such differences are unknown, but perhaps species, blood vessel, and/or experimental differences account for such differences in the observations made. It is clear from the present study that endothelium-dependent relaxation of bovine intrapulmonary artery and vein occurred unimpared in the absence of extracellular magnesium but that extracellular calcium was required for relaxation. The bioassay cascade technique revealed clearly that endothelium-dependent vasodilators generate or release EDRF in the absence of perfused magnesium but not in the absence of perfused calcium. On the other hand, endothelium-independent relaxation elicited by glyceryl trinitrate or nitroso compounds occurred independent of extracellular magnesium or calcium.

The present study does not address the cellular site of interaction of magnesium and calcium. Evidence exists for the antagonistic actions of magnesium and calcium at both calcium and magnesium transport sites associated with the vascular smooth muscle membrane. Recently, the existence of calcium channels in isolated endothelial cells has been suggested, which may become important in magnesium-calcium interactions.

Magnesium and calcium serve to regulate vascular smooth muscle tone. Calcium influx into the cytosol causes smooth muscle contraction, whereas magnesium can control calcium influx into the cell by competitively interacting at transport sites. A decrease in extracellular magnesium concentration is associated with ischemic heart disease and hypertension, whereas infusions of magnesium produce vasodilation as a result of reduced calcium influx. The present study provides evidence that the vascular endothelium, through the actions of EDNO, is responsive to changes in the extracellular concentration of magnesium and calcium and that this may protect against vasoconstriction.

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