Endothelium-Dependent Modulation of cGMP Levels and Intrinsic Smooth Muscle Tone in Isolated Bovine Intrapulmonary Artery and Vein

Louis J. Ignarro, Russell E. Byrns, and Keith S. Wood

The role of the endothelium in modulating cyclic nucleotide levels and intrinsic smooth muscle tone was studied in isolated rings of bovine intrapulmonary artery and vein. Cyclic 3',5'-guanosine monophosphate (cGMP) levels were threefold to fourfold higher in unrubbed artery and vein than in vessels that had been denuded of endothelium. Cyclic 3',5'-adenosine monophosphate (cAMP) levels were twofold higher in unrubbed than in endothelium-denuded artery, but no differences were observed in veins. Methylene blue, an inhibitor of guanylate cyclase, decreased cGMP but not cAMP levels, and this was accompanied by increases in smooth muscle tone. M&B 22,948, an inhibitor of cGMP-phosphodiesterase, increased cGMP but not cAMP levels, and this was accompanied by decreases in smooth muscle tone. Unrubbed vessels were more sensitive than endothelium-denuded vessels to the actions of both methylene blue and M&B 22,948, and this may be attributed to endothelium-dependent increases in cGMP turnover. Moreover, unrubbed vessels were more sensitive than endothelium-denuded vessels to contractile responses to phenylephrine and potassium, and these responses were potentiated by methylene blue and attenuated by M&B 22,948. Although indomethacin lowered cAMP levels in unrubbed artery, no changes in tone or contractile responsiveness were observed. A consistent observation was that the smaller branches of unrubbed but not endothelium-denuded intrapulmonary artery and vein had higher levels of cGMP but not cAMP, were sensitive to endothelium-dependent vasodilators, were more sensitive to methylene blue, and would not maintain a steady level of submaximal tone to phenylephrine when compared with larger branches from a common vascular bed. These data indicate that endothelium-derived factors in intrapulmonary artery and vein markedly influence intrinsic cGMP levels, sensitivity to endothelium-dependent vasodilators, smooth muscle tone, and contractile responsiveness. (Circulation Research 1987;60:82-92)

The principal objective of the present study was to determine the relation between alterations in intrinsic cyclic nucleotide levels and smooth muscle tone as a function of endothelial integrity in isolated rings of bovine intrapulmonary artery and vein. Moreover, rings prepared from arterial and underlying venous segments of varying diameters isolated from the pulmonary vascular bed were compared with respect to cyclic nucleotide levels and sensitivities to contractile and relaxant agents. This approach is different from that of previous studies from this and other laboratories where the focus was on the stimulation of cyclic 3',5'-guanosine monophosphate (cGMP) accumulation by added endothelium-dependent vasodilators. Agents such as acetylcholine, arachidonic acid, calcium ionophores, tissue hormones, and polypeptides have been shown to cause endothelium-dependent arterial relaxation that was associated temporally with cGMP accumulation. In-depth reports on the relation between arterial cGMP levels and vascular smooth muscle tone are lacking. In addition, very little information is available on the relation between vascular endothelium and arterial cyclic nucleotide levels in the absence of added vasodilators. Even less is known about these relations in vein.

Studies conducted exclusively with vascular smooth muscle have generally supported the view that cGMP mediates or modulates the relaxant responses to nitrogen-oxide-containing and endothelium-dependent vasodilators. This hypothesis has been strengthened by the observations that methylene blue, an inhibitor of soluble guanylate cyclase, markedly and selectively inhibited both relaxation and cGMP accumulation elicited by vasodilators that stimulate vascular cGMP formation. Moreover, the selective inhibitor of cGMP-specific phosphodiesterase, M&B 22,948, was reported to potentiate vascular smooth muscle relaxant responses to agents that elevate vascular levels of cGMP. Several recent reports suggest that vascular endothelial factors play a major role in influencing local smooth muscle concentrations of both cGMP and cyclic 3',5'-adenosine monophosphate (cAMP). In the present study, a detailed analysis was made of the influence of endothelium, methylene blue, M&B 22,948, and indomethacin on both cyclic nucleotide levels and intrinsic smooth muscle tone in intrapulmonary artery and vein. In addition, the effects of pre-treatment of vascular rings with methylene blue or M&B 22,948 on subsequent contractions produced by phenylephrine or potassium were determined. In view of repeated preliminary observations that phenyleph-
rime-elicted contractions or tone of arterial rings prepared from intrapulmonary branches of small diameter were markedly impaired, we studied the relation among endothelium, cGMP levels, and contractile responses in artery and vein of various diameters. The rationale for these experiments was the possibility that cGMP levels, and therefore smooth muscle tone, are influenced by endothelium-dependent relaxing factors both in artery and vein and that smaller blood vessels may possess greater amounts and/or activities of such factors.

Materials and Methods

Preparation of Arterial and Venous Rings

Bovine lungs were obtained from 2-10-year-old animals and transported to the laboratory as described previously. Transmission time was reduced, however, from 2 hours to 30 minutes. Intrapulmonary arterial and venous branches were rapidly isolated and gently cleaned of parenchyma, fat, and adhering connective tissue and placed in cold preoxygenated buffered Krebs-Ringer’s solution. In most experiments, segments of the 2nd arterial branch and underlying 2nd venous branch extending into the larger lobe were isolated. Outside diameters were 4-5 mm (artery) and 6-7 mm (vein). In certain experiments as indicated, segments of the 3rd and 4th vessel branches from larger lungs (older cows) were isolated (diameters are given in the text). Vessels were sliced into rings as described previously. Rings prepared in this manner possessed an intact endothelium assessed by 90-100% relaxation responses to \(10^{-7}\) M acetylcholine (arteries) or \(10^{-8}\) M bradykinin (veins). Such vascular rings are referred to in the text as unrubbed. Endothelial cells were largely removed from vessel segments prior to slicing by gently passing a moistened stick (from a cotton swab) into the lumen and rubbing against the intima for 30-40 seconds without stretching the vessel walls. Rubbed or endothelium-denuded rings sharply contracted in response to acetylcholine (arteries) or bradykinin (veins).

Recording of Muscle Tension

Arterial and venous segments were rapidly sliced into rings (4-mm wide) using a specially designed microtome. Rings were mounted by means of fine nichrome wires in jacketed, 25-ml capacity, drop-away chambers containing Krebs-Ringer’s (37°C) gassed with 95% \(\text{O}_2\)-5% \(\text{CO}_2\). The upper nichrome wire of each ring was attached to a force-displacement transducer (Grass Instrument Co., Quincy, Mass., Model FT03C) and changes in isometric force were recorded on a Grass Polygraph (Model 79D). Length-tension relations were determined initially for each size (diameter) of unrubbed and endothelium-denuded ring of artery and vein employed in this study and were performed in the following manner: Tension was adjusted to the optimal length for maximal isometric contractions to potassium by progressively stretching the rings and repeatedly obtaining contractile responses to 80 mM KCl, with washing and 15 minutes of equilibration between each contractile response. Individual relations for each ring did not change appreciably after a 90-minute period of equilibration following the initial set of responses. In general, the larger the ring diameter the greater the optimal length (tension) required for maximal isometric contraction. Arterial rings usually required a greater optimal tension than venous rings of comparable diameters. Optimal tensions for arterial and venous rings did not vary significantly as a function of intimal rubbing (presence or absence of endothelium). The above relation was obtained for 6-8 rings of each diameter of rubbed and unrubbed artery and vein. The optimal tensions determined in these initial experiments were employed in all subsequent experiments. Rings with diameters falling outside the ranges tested were not studied. Optimal tensions and maximal contractile tensions (ranges) developed in response to KCl at optimal lengths (both expressed in grams) were as follows: artery—2nd branch (6; 20-24), 3rd branch (4; 17-20), 4th branch (3; 12-15); vein—2nd branch (4; 18-22), 3rd branch (3; 16-19), 4th branch (2; 12-16).

Bovine intrapulmonary arterial and venous rings were routinely depolarized by the addition of 120 mM KCl following 2 hours of equilibration at optimal tension and then washed and allowed to equilibrate for 45 minutes prior to initiating any given protocol. This procedure increases and stabilizes any subsequent submaximal precontractile responses to phenylephrine, presumably by loading the smooth muscle cells with calcium. This procedure has been employed routinely for bovine pulmonary vessels in this laboratory for several years.

Procedures Involving Methylene Blue and M&B 22,948

In contrast to phenylephrine and potassium, which produce rapidly developing contractions, methylene blue elicited more slowly developing contractile responses. Similarly, in contrast to acetylcholine and glyceryl trinitrate, which produce rapidly developing relaxations, M&B 22,948 elicited more slowly developing relaxant responses. In view of these properties, it was necessary to allow either methylene blue or M&B 22,948 to interact with vascular rings for 15 minutes before freeze-clamping the rings for cyclic nucleotide analysis. Time-course experiments revealed that 15 minutes was the optimal time for freeze-clamping because maximal or near-maximal changes in cyclic nucleotide levels occurred at that time (Table 1). This time interval is much longer than the 30-90-second intervals usually employed in this laboratory for more rapidly acting agents.

Determination of Cyclic Nucleotide Levels

All cGMP and cAMP determinations were made on arterial and venous rings that had been equilibrated under tension and depolarized with KCl. Indeed, tone was monitored in all rings until the time of freeze-clamping. The use of special drop-away bath chambers, freeze-clamping of rings, preparation and extrac-
tion of tissues for cyclic nucleotide determinations, and radioimmunoassay procedures were described previously. 2,6,11,12,16 cGMP and cAMP levels were determined in aliquots from the same ring extract. None of the test agents added to bath chambers directly interfered with antigen-antibody binding in the radioimmunoassay procedures. Recoveries of standard amounts of added cyclic nucleotides were determined periodically, and the values ranged from 93-102%. Therefore, no corrections for sample recoveries were made.

**Chemicals and Solutions**

Acetylcholine chloride, phenylephrine hydrochloride, bradykinin triacetate, methylene blue, and indomethacin were obtained from Sigma Chemical Co., St. Louis, Mo. Glycerol trinitrate (10% w/w triturate in lactose) was a gift from ICI Americas, Inc., Wilmington, Del., and M&B 22,948 was generously provided by May and Baker Ltd., Dagenham, Essex, UK. Solutions of hygroscopic acetylcholine chloride were prepared, aliquoted, and stored frozen as described previously. 5 Bradykinin is unstable and was prepared fresh in distilled water just before use. Stock indomethacin solutions (10 mM) were prepared fresh in 100 mM NaHCO₃ and diluted with buffered Krebs-Ringer’s solution to the desired concentrations. M&B 22,948 was dissolved in 0.1N NaOH to a final concentration of 10 mM and stored at 4°C; dilutions in buffered Krebs-Ringer’s solution were made just before use. All other drugs were prepared fresh in water just before use. Buffered Krebs-Ringer’s solution consisted of (in mM) NaCl 118, KCl 4.7, CaCl₂ 1.5, NaHCO₃ 25, MgSO₄ 1.1, KH₂PO₄ 1.2, and glucose 5.6. Depolarizing KCl solution had a composition similar to buffered Krebs-Ringer’s solution except the NaCl was replaced with an equimolar concentration of KCl. The salt solution in which the bovine lungs were transported from the slaughterhouse to the laboratory (30 minutes) had the following composition (in mM): Tris HCl (pH 7.4) 23.8, NaCl 125, KCl 2.7, CaCl₂ 1.8, and glucose 11. Lungs were excised from cows and immediately submerged in ice cold salt solution contained in a plastic bag. The bag was sealed, placed in a cooler packed with ice, and brought to the laboratory.

**Calculations and Statistical Analysis**

Muscle contraction was measured as the increase in tension above the initial adjusted optimal tension as indicated. Relaxation was measured as the decrease in tension either below the adjusted initial value or below the elevated tension elicited by precontracting the muscle with phenylephrine as indicated. Values are expressed as the mean ± SE and represent unpaired data. Comparisons were made using either the Duncan’s multiple range test, 26 where comparisons with a common control were made (Figures 1,2,4,5), or the Student’s t test for unpaired values for all other comparisons. The level of statistically significant difference was p < 0.05.

**Results**

**Influence of Endothelium on Alterations by Methylene Blue of Intrinsic Tension and cGMP Levels in Intrapulmonary Artery and Vein**

Methylene blue elicited a concentration-dependent increase in tension of intrapulmonary arterial rings that was associated with a concomitant and parallel decrease in levels of cGMP (Figure 1). Tension increased from control values of 6 grams to about 14 grams in endothelium-intact arterial rings. The increase in tension of intrapulmonary arterial rings that was associated with a concomitant and parallel decrease in levels of cGMP (Figure 1). Tension increased from control values of 6 grams to about 14 grams in endothelium-intact arterial rings. The increase in tension of intrapulmonary arterial rings that was associated with a concomitant and parallel decrease in levels of cGMP (Figure 1). Tension increased from control values of 6 grams to about 14 grams in endothelium-intact arterial rings. The increase in tension of intrapulmonary arterial rings that was associated with a concomitant and parallel decrease in levels of cGMP (Figure 1). 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methylene blue. Endothelium-denuded rings were clearly less sensitive than endothelium-intact rings to methylene blue (Figure 1). Tension increased to a maximum of about 8.5 grams and cGMP levels decreased from a markedly reduced initial level of 8–9 pmol/g tissue to about 3 pmol/g tissue. Thus, removal of the endothelium resulted in a fourfold decrease in arterial levels of cGMP, which were further decreased about threefold by $10^{-4} \text{M}$ methylene blue. The same concentration of methylene blue produced a sevenfold to eightfold decrease in cGMP levels in unrubbed arterial rings. Denuded rings were clearly less sensitive than unrubbed rings to M&B 22,948. Phenylephrine did not significantly alter cGMP levels.

Veins behaved similarly to arteries with respect to the effects of M&B 22,948 except that the veins were less sensitive and showed less marked responses (Figure 5). Regardless of differences in sensitivities or responsiveness between arterial and venous rings, a good correlation was observed between decreases in tension and increases in cGMP accumulation in veins produced by M&B 22,948. In the time-course experiments shown in Table 1, a tenfold higher concentration of methylene blue was used in endothelium-denuded than unrubbed rings because of the differences in sensitivities of the preparations to methylene blue.
FIGURE 3. Effect of M&B 22,948 on tension and phenylephrine-elicited precontraction of unrubbed bovine intrapulmonary artery. Top tracing: cumulatively increasing concentrations of M&B 22,948 were added to arterial rings adjusted to an optimal tension of 6 grams. Bottom tracing: cumulatively increasing concentrations of M&B 22,948 were added to phenylephrine (PE; $10^{-5}$ M)-precontracted arterial rings adjusted to an optimal tension of 6 grams. Concentrations are illustrated as exponents to the base power 10 and represent final bath concentrations. One typical example from 6 experiments is illustrated. Cows were 5 years old.

Effects of Methylene Blue, M&B 22,948, and Indomethacin on cAMP Levels in Intrapulmonary Artery and Vein

cAMP determinations were made in aliquots of the same tissue extracts employed to analyze cGMP in the experiments illustrated in Figures 1, 2, 4, and 5. Only those rings receiving no addition (control) or $10^{-5}$ M concentrations of methylene blue or M&B 22,948 were analyzed for cAMP. In addition, rings exposed to $10^{-5}$ M indomethacin were analyzed. The data are illustrated in Table 2. One significant observation was that unrubbed arterial rings had twofold higher levels of cAMP than did endothelium-denuded arterial rings. Venous rings, on the other hand, did not show any significant influence of endothelium on cAMP levels. cAMP levels were twofold to fourfold lower in veins than in arteries. Neither methylene blue nor M&B 22,948 significantly altered cAMP levels, but indomethacin consistently reduced cAMP levels almost twofold in unrubbed arterial rings (Table 2). As shown in the next section, indomethacin failed to influence arterial tension. Indomethacin did not significantly alter cAMP levels in veins or in denuded arteries. Although not shown, cGMP levels were not changed in any vessel type by indomethacin.

FIGURE 4. Effect of M&B 22,948 on tension and cGMP levels in phenylephrine-precontracted bovine intrapulmonary artery isolated with (+ E) or without (− E) an intact endothelium. An optimal tension of 6 grams was applied to all arterial rings. Submaximal contractions were elicited by $10^{-4}$ M phenylephrine, and M&B 22,948 was added at peak contractile responses. Rings were freeze-clamped 15 minutes after addition of M&B 22,948. Values represent the mean ± SE using 6–8 rings isolated from 3 to 4 animals (2 rings per animal). Cows were 5 years old. *Significantly different ($p < 0.05$) from corresponding value in the absence of M&B 22,948.

FIGURE 5. Effect of M&B 22,948 on tension and cGMP levels in phenylephrine-precontracted bovine intrapulmonary vein isolated with (+ E) or without (− E) an intact endothelium. An optimal tension of 4 grams was applied to all venous rings. Submaximal contractions were elicited by $10^{-4}$ M phenylephrine, and M&B 22,948 was added at peak contractile responses. Rings were freeze-clamped 15 minutes after addition of M&B 22,948. Values represent the mean ± SE using 6 to 8 rings isolated from 3 to 4 animals (2 rings per animal). Cows were 5 years old. *Significantly different ($p < 0.05$) from corresponding value in the absence of M&B 22,948.
Influence of Methylene Blue, M&B 22,948, and Indomethacin on Phenylephrine-Elicited Contraction of Intrapulmonary Artery

In addition to the action of methylene blue to directly contract unrubbed arteries and veins, low concentrations of methylene blue, which produced little or no contractile effects (Figures 1 and 2), markedly enhanced the contractile response to phenylephrine (Figure 6). The concentration-response curve for phenylephrine was shifted to the left and upward by 10^{-6} M methylene blue. On the other hand, 10^{-5} M indomethacin (Figure 6). Indomethacin (10^{-5} M) produced no effect on phenylephrine-elicited contractions. Thus, although indomethacin significantly lowered arterial cAMP levels (Table 2), no accompanying alteration of contractile responses was evident. The responses to methylene blue and M&B 22,948 in endothelium-denuded arterial rings were much less than those observed in unrubbed rings (not shown). Similarly, unrubbed venous rings showed qualitatively similar but less marked responsiveness than did the arterial rings to methylene blue and M&B 22,948.

Influence of Endothelium on Contractile Responses to Phenylephrine in Intrapulmonary Artery of Various Diameters

Repeated experiments with unrubbed arterial and venous rings prepared from smaller arterial segments obtained from the 2nd branch of intrapulmonary artery. Endothelium-complete rings prepared from this arterial segment showed good contractile responses to phenylephrine without any significant impairment of tone at peak contractile responses (Figure 7; Tracing A). Endothelium-denuded arterial rings were less sensitive and showed less marked contractile responses, as reported previously. Unrubbed rings prepared from smaller arterial segments obtained from the 3rd and 4th branches (Figure 7; Tracings B and C) showed a great loss in tone after phenylephrine-elicited contractions. Potassium, however, produced large contractions that were characterized by no significant loss of tone. These observations suggest that the smaller arterial branches generate either greater amounts of, or a more active, endothelium-derived relaxing factor than do the larger branches. Consistent with this view is the finding that endothelium-denuded arterial rings prepared from identical smaller branches did not display any significant loss of tone during phenylephrine-elicited contractions (Figure 7). Moreover, denuded
artery + E

artery - E

FIGURE 7. Differences in contractile responses to phenylephrine and KCl among varying diameters of bovine intrapulmonary artery isolated with (+E) or without (-E) an intact endothelium. Tracing a: 2nd branch, 4 mm diameter; tracing b: 3rd branch, 2.5 mm diameter; tracing c: 4th branch, 1.5 mm diameter. Optimal tensions in grams were 6 (tracing a), 4 (tracing b), and 3 (tracing c). Cumulatively increasing concentrations of phenylephrine (PE) or KCl were added as indicated. PE concentrations are illustrated as exponents to the base power 10 while KCl concentrations are expressed as mM. All concentrations represent final bath concentrations. The KCl responses were conducted in the same arterial rings (tracing c) that received PE (after washing and 45-minute equilibration). One typical example from 8 experiments is illustrated. Cows were 6–10 years old.

arterial rings showed greater maximal contractile responses to phenylephrine with decreasing diameters of the arterial segment. It is possible, however, that a similar relation between diameter and contractile responses to phenylephrine existed also in unrubbed artery, but this could not be readily observed due to the rapid loss of tone. Although endothelium integrity did not influence the maximal contractile response to KCl (30 mM), the endothelium appeared to increase slightly the sensitivity of the arterial rings to KCl, for example, at the smaller concentrations (Figure 7). Venous rings prepared from smaller branches showed properties that were similar to those of arterial rings (some data shown in Figure 8).

Effect of Methylene Blue on Phenylephrine-Elicited Contraction and Endothelium-Dependent Relaxation in Small Branches of Intrapulmonary Artery and Vein

Unrubbed rings were prepared from the 3rd branches of intrapulmonary artery and vein. Both arterial and venous rings showed marked impairment of tone maintenance after contraction by phenylephrine (Figure 8). Acetylcholine and bradykinin produced marked relaxation of precontracted artery and vein, respectively. Indeed, unrubbed rings prepared from the small arterial and venous branches were consistently about tenfold more sensitive than those from larger branches to the relaxant effects of acetylcholine (artery) and bradykinin (artery and vein). However, the extent of the contribution of the inherent loss of tone to these drug-elicited relaxant responses was difficult to assess, and thus the relaxant responses could not be quantified. Methylene blue pretreatment of the rings abolished the loss of tone and enhanced the contractile responses to phenylephrine. In this regard, the methylene-blue-pretreated, unrubbed rings showed remarkably similar properties to those of endothelium-denuded rings. Moreover, the methylene-blue-pretreated rings showed only very small relaxant responses to acetylcholine or bradykinin (Figure 8), as reported previously.5, 6, 37

Effect of M&B 22,948 on Phenylephrine-Elicited Contraction in Endothelium-Denuded Small Branches of Intrapulmonary Artery and Vein

Endothelium-denuded rings were prepared from the 3rd branches of intrapulmonary artery and vein. Both arterial and venous rings maintained tone after contraction elicited by phenylephrine (Figure 9). Pretreatment of both rings with 10^{-5} M M&B 22,948 for 15 minutes, however, resulted in the development of marked impairment of tone maintenance after contraction elicit-
Effect of M&B 22,948 on phenylephrine-elicited contractions in endothelium-denuded (−E) bovine intrapulmonary artery (top tracings) and vein (bottom tracings). The 3rd branches of artery (2.5 mm diameter) and vein (3.5 mm diameter) were used to prepare rings. Optimal tensions in grams were 4 (artery) and 3 (vein). Concentrations of phenylephrine (PE) and M&B 22,948 (M&B) are illustrated as exponents to the base power 10 and represent final bath concentrations. After the first set of responses to phenylephrine, rings were washed and equilibrated for 45 minutes prior to initiating the second set of responses. M&B was added to bath chambers 15 minutes prior to starting the second set of responses to phenylephrine. One typical example from 6 experiments is illustrated. Cows were 6–10 years old.

Differences in cGMP Levels Among Various Branches of Unrubbed Intrapulmonary Artery and Vein

The progressive loss of phenylephrine-elicited tone in unrubbed rings prepared from smaller arterial and venous branches prompted an analysis of intrinsic cGMP levels in these branches as elevated vascular levels of cGMP are often associated with decreased contractile responses and with endothelium-dependent relaxant responses. A consistent and inverse relation between vessel diameter and cGMP levels was observed in unrubbed arteries and veins (Table 3). Methylene blue markedly lowered cGMP levels in both artery and vein. Endothelium-denuded arterial rings had much lower cGMP levels, which did not vary significantly among branches. Similar observations were made in endothelium-denuded venous rings.

Discussion

The vascular endothelium markedly influenced intrinsic or basal levels of cGMP, and cAMP to a much lesser extent, in isolated rings of bovine intrapulmonary artery and vein. cGMP levels were twofold to threefold higher in unrubbed than in endothelium-denuded vascular rings. cAMP levels were twofold high-

Table 3. Differences in Levels of cGMP Among Arterial and Venous Rings of Various Diameters and Effects of Methylene Blue

<table>
<thead>
<tr>
<th>Vessel Branch</th>
<th>Control</th>
<th>10⁻⁵ M Methylene Blue</th>
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<tbody>
<tr>
<td></td>
<td>+E*</td>
<td>−E</td>
</tr>
<tr>
<td>Artery</td>
<td></td>
<td></td>
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<tr>
<td>Small lung</td>
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<tr>
<td>2nd</td>
<td>20 ± 1.8</td>
<td>6.2 ± 0.5</td>
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<tr>
<td>3rd</td>
<td>42 ± 2.8</td>
<td>7.4 ± 0.6</td>
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<tr>
<td>4th</td>
<td>53 ± 3.2</td>
<td>8.8 ± 0.8</td>
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<tr>
<td>Large lung</td>
<td></td>
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<tr>
<td>2nd</td>
<td>31 ± 2.0</td>
<td>9.1 ± 0.8</td>
</tr>
<tr>
<td>3rd</td>
<td>42 ± 2.8</td>
<td>7.4 ± 0.6</td>
</tr>
<tr>
<td>4th</td>
<td>53 ± 3.2</td>
<td>8.8 ± 0.8</td>
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<tr>
<td>Vein</td>
<td></td>
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<tr>
<td>Small lung</td>
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<tr>
<td>2nd</td>
<td>11 ± 0.7</td>
<td>3.4 ± 0.5</td>
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<tr>
<td>3rd</td>
<td>28 ± 1.7</td>
<td>5.7 ± 0.7</td>
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<tr>
<td>4th</td>
<td>41 ± 2.5</td>
<td>5.2 ± 0.9</td>
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<td>Large lung</td>
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<tr>
<td>2nd</td>
<td>18 ± 1.6</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>3rd</td>
<td>28 ± 1.7</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>4th</td>
<td>41 ± 2.5</td>
<td>5.2 ± 0.9</td>
</tr>
</tbody>
</table>

Arterial and venous rings that were unrubbed (+E) or endothelium-denuded (−E) were mounted under optimal tension as described in the text, allowed to equilibrate for 2 hours, and depolarized with KCl. After washing and 45 minutes of equilibration, methylene blue was added for 15 minutes as indicated. Phenylephrine was then added to all rings at concentrations predetermined to elicit contractile responses equivalent to 65–75% of maximal. At peak contractile responses, the rings were quick-frozen, extracted, and assayed for cGMP. Small lung signifies cows that were 2 years old. Large lung signifies cows that were 5 years old. Values represent the mean ± SE using 8–10 rings isolated from 4–5 animals (2 rings per animal).

*Values for each arterial branch (+E) are significantly different (p < 0.05) from one another. Values for each venous branch (+E) are significantly different (p < 0.05) from one another.
†All values for +E are significantly different (p < 0.05) from corresponding values obtained in the absence of methylene blue (control).
er in unrubbed than in denuded artery, but no difference was found in veins. Furthermore, a good inverse correlation was found between the diameter of unrubbed intrapulmonary vessels and cGMP but not cAMP levels. Thus, smaller branches arising from the main intrapulmonary artery and vein displayed significantly higher cGMP levels than the larger branches. In contrast, cGMP levels remained relatively constant in endothelium-denuded branches. These observations support and strengthen the view that endothelium-derived substances are capable of increasing vascular concentrations of cGMP. Indeed, we have recently found that soluble guanylate cyclase purified from bovine lung is directly activated by endothelium-derived substances.

The above observations indicate that the smaller intrapulmonary vessels generate greater amounts of, or possess more active, endothelium-derived factors capable of elevating cGMP levels than the larger vessels. These endothelium-derived factors appear to be the putative smooth muscle relaxing factors first described by Furchgott and Zawadzki. Evidence for this view that the smaller arterial rings were more sensitive than larger rings to endothelium-dependent relaxants such as acetylcholine and bradykinin. Moreover, phenylephrine-elicited submaximal tone was impaired and could not be maintained in the smaller vessel branches when the endothelium was intact. The smaller the vessel the faster the loss of contractile tone preinduced by phenylephrine. On the other hand, denuded rings of all diameters tested maintained tone. Moreover, unrubbed rings maintained tone in response to potassium. Although potassium-precontracted, unrubbed arterial rings relax in response to endothelium-dependent vasodilators, the magnitude of such relaxant responses is markedly attenuated when compared to responses in rings precontracted with phenylephrine, norepinephrine, and related receptor agonists. The reason for this difference is unknown, but may be attributed to an inhibitory action by potassium on the vascular responsiveness to, or generation of, endothelium-derived relaxing factors.

One clear relation observed in unrubbed vessels was a good correlation between elevated levels of cGMP and the inability to maintain submaximal contractile tone to phenylephrine. Methylene blue lowered cGMP levels and restored the capacity of rings to maintain tone. Indeed, increasing concentrations of methylene blue caused further depletion of cGMP accompanied by the development of contractile responses. In denuded rings, where cGMP levels were low and tone was well maintained, high concentrations of methylene blue further depressed cGMP levels and caused contractions. Methylene blue was previously reported to augment tone slightly in endothelium-denuded coronary and intrapulmonary artery, to enhance acetylcholine-elicited contractions in endothelium-denuded arterial preparations, and to augment phenylephrine-elicited contractions in unrubbed arterial rings. M&B 22,948, a selective inhibitor of cGMP-phosphodiesterase, caused a concentration-dependent increase in cGMP levels and a further decrease in the capacity of either unrubbed or endothelium-denuded rings to maintain tone. These observations demonstrate clearly the existence of an inverse relation between cGMP levels and maintenance of tone preinduced by receptor-sensitive contractile agents. Thus, the endothelium may control vascular tone indirectly by influencing cGMP levels in smooth muscle, especially in the presence of elevated tone.

The present findings are highly consistent with the view that cGMP plays an important role in expressing the vascular smooth muscle relaxant effects of numerous endothelium-dependent and independent vasodilators (see reviews by Furchgott and Ignarro and Kadowitz). Almost all previous reports have examined the relation between vasodilator-stimulated cGMP accumulation and smooth muscle relaxation. In the present study, alterations in intrinsic cGMP levels resulted in compatible changes in contractile tone. The single most important factor in the influence of arterial tone in the isolated rings by cGMP appears to be the vascular endothelium. Endothelium-derived relaxing factors are likely responsible for the stimulation of cGMP accumulation. The extent to which the endothelium influences cGMP levels and tone apparently varies with the diameter of the vessel, at least in bovine intrapulmonary artery and vein. The smaller branches were much more sensitive than the larger vessels to the influence of the endothelium. On the basis of these observations one might speculate that small resistance and capacitance vessels may be more actively influenced than large vessels by endothelium-dependent relaxing factors released by endogenous vasoreactive substances such as autacoids.

Little information is available on the importance of the endothelium in venous responses to vasodilators. Endothelium-dependent arterial relaxants generally do not relax, and in many cases, contract endothelium-intact venous preparations (see reviews by Furchgott and Ignarro and Kadowitz). Bradykinin, however, was recently shown to relax bovine intrapulmonary vein and elevate cGMP levels by endothelium-dependent mechanisms. On the other hand, veins are more sensitive than arteries to the cGMP accumulating and relaxant effects of the nitrogen-oxide-containing, endothelium-independent vasodilators. We have found that, of the many endogenous agents tested, only bradykinin caused endothelium-dependent cGMP accumulation in, and relaxation of, bovine intrapulmonary vein. In addition, venous endothelial factors were capable of directly activating soluble guanylate cyclase. In the present study, unrubbed venous rings had higher resting levels of cGMP than did denuded rings, and the smaller unrubbed venous branches had higher cGMP levels than did the larger vessels. Contractile tone in response to phenylephrine was also less easily maintained in the smaller venous rings but quantitatively these differences were not as marked as those observed in arterial rings. In general, cGMP levels were slightly but significantly higher in artery than in vein. Thus, veins possess endothelium-derived relax-
ing factors but venous endothelial cells apparently lack selective receptors for vasodilators, such as acetylcholine, that relax primarily artery. The physiologic relevance of such differences between artery and vein is presently unknown. More in vivo studies are required to answer this question.

cAMP levels were markedly higher than cGMP levels in both arteries and veins regardless of endothelial integrity. The endothelium, however, influenced cAMP levels only in artery. Denudation of arterial rings resulted in a twofold decrease in cAMP levels, as did pretreatment of unrubbed rings with indomethacin. However, indomethacin did not significantly alter either intrinsic tension or contractile responsiveness to phenylephrine. Therefore, endothelium-dependent alterations in arterial cAMP levels do not appear to be an important factor in influencing vascular smooth muscle tone. The mechanism by which indomethacin causes endothelium-dependent reductions in arterial cAMP levels may be attributed to inhibition of prostacyclin formation. Indomethacin also partially antagonizes the endothelium-dependent, cAMP-mediated component of arterial relaxation in response to arachidonic acid presumably by inhibiting prostacyclin formation. Indomethacin was reported to inhibit both endothelium-dependent relaxation and 6-keto PGF₁α formation in canine femoral artery elicited by arachidonic acid. Bovine intrapulmonary veins do not relax or show increases in cAMP levels in response to either arachidonic acid or prostacyclin, and this could account for the lack of influence of endothelium on venous cAMP levels.

This laboratory has consistently found that unrubbed rings of intrapulmonary artery and vein are more responsive than are endothelium-denuded rings to contractile agents, including phenylephrine, U46619, and potassium. A ready explanation of these observations is not yet forthcoming. On the other hand, the increased responsiveness of unrubbed rings to the contractile effects of methylene blue may be attributed to a greater turnover of cGMP in unrubbed than in denuded rings. cGMP levels are several times higher in unrubbed rings, and such preparations are also more sensitive than denuded rings to the relaxant actions of M&B 22,948. Thus, by inhibiting cGMP formation and degradation, respectively, methylene blue and M&B 22,948 might be expected to show greater sensitivity in unrubbed vessels. Contractile agents such as phenylephrine and potassium did not alter cyclic nucleotide levels, however, and so alternative mechanisms are involved. Moreover, certain unrubbed arterial preparations from other tissues and species may be less sensitive than endothelium-denuded vessels to contractile agents. Such observations are opposite to those reported here and, therefore, additional endothelium-derived substances present in some vessels, but not in others, may be important in influencing contractile responsiveness to α₁-receptor agonists, potassium, and perhaps other agents.

In conclusion, the present study demonstrates that the vascular endothelium in bovine intrapulmonary artery and vein exhibits a marked influence on smooth muscle levels of cGMP. These alterations are reflected as significant changes in smooth muscle responsiveness to contractile agents, including sensitivity and maintenance of contractile tone. The consequence of elevated levels of cGMP appears to be an impairment in the ability to maintain a steady level of submaximal tone. The mechanism by which the endothelium raises cGMP levels is through endothelium-derived factors that directly activate soluble guanylate cyclase in the smooth muscle. Thus, endothelium-derived factors may play an important regulatory role in influencing vascular smooth muscle tone by directly altering levels of cGMP.

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