Measurement of Endothelial Cytosolic Calcium Concentration and Nitric Oxide Production Reveals Discrete Mechanisms of Endothelium-Dependent Pulmonary Vasodilatation

Stephen L. Archer and Nancy J. Cowan

Nitric oxide is an endothelium-derived relaxing factor. Conversion of L-arginine to nitric oxide follows mediator-induced elevation of endothelial cytosolic calcium concentration. However, not all endothelium-dependent vasodilatation is caused by endothelium-derived relaxing factor, and few studies have correlated changes in vascular tone with measurement of free cytosolic calcium concentration or nitric oxide. The effects of three endothelium-dependent vasodilators (acetylcholine, bradykinin, and A23187) on vascular tone and nitric oxide production were studied in proximal rat pulmonary artery rings. Changes in free cytosolic calcium concentration and nitric oxide production were also studied in bovine pulmonary artery endothelial cells. A23187 and bradykinin caused pulmonary vasodilatation, nitric oxide production, and elevation of endothelial calcium concentrations. Although acetylcholine caused endothelium-dependent vasodilatation, it reduced free cytosolic calcium concentration and failed to increase nitric oxide levels. Acetylcholine-induced dilatation was partially inhibited by meclofenamate but was unaffected by ouabain. Acetylcholine, unlike bradykinin and A23187, does not act through a nitric oxide-dependent mechanism in the rat pulmonary vasculature. (Circulation Research 1991;68:1569–1581)

The endothelium can mediate pulmonary vasodilatation1–4 by production of prostanoids, endothelium-derived relaxing factors (EDRFs), such as nitric oxide,5,6 and an endothelium-derived "hyperpolarizing factor" (EDHF).7,8 An apparently simple hemodynamic phenomenon may be the summation of a number of these endothelium-based mechanisms acting in concert. The relative contribution of these mechanisms to a vasodilator response may vary, depending on the dose of drug administered and the species and vascular bed studied.9–13 This complexity underscores the need to correlate hemodynamic changes with concurrent measurement of cell biochemistry. The few studies6,14–17 that have correlated endothelium-dependent vasodilator–induced nitric oxide production with changes in vascular tone have been conducted on systemic vessels. In the lung, the identity and physiological importance of EDRF have primarily been evaluated through the use of EDRF inhibitors and bioassays, which are of varying specificity.1–4,13,16–20 Such studies show sufficient discrepancies between the hemodynamic profiles of nitric oxide and the many endothelium-dependent vasodilators to cast doubt on the hypothesis that all endothelium-dependent pulmonary vasodilators act through a nitric oxide–dependent mechanism. For example, although bradykinin (BK) and acetylcholine (ACh) cause endothelium-dependent pulmonary vasodilatation in the lung,3 the potency, duration of action, and predisposition to tachyphylaxis of these agents differ from each other and from exogenous nitric oxide.3 N0-Monomethyl L-arginine (LNMA), a competitive inhibitor of the conversion of L-arginine to nitric oxide, inhibits BK- but not ACh-induced pulmonary vasodilatation.2 Furthermore, Menon et al21 showed that A23187- but not ACh-induced pulmonary vasodilatation was associated with nitric oxide synthesis in bovine pulmonary arteries. Since EDRF bioactivity is thought to be associated with a rise in endothelial free cytosolic calcium concentration22–24 and the subsequent conversion of L-arginine to

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Denuded Pulmonary Artery Rings

Denuded PA rings (two rings per bath, n=6) were constricted with norepinephrine (3.1×10^-6 M) and then challenged with ACh (10^-5−10^-3 M). The bath was flushed, SOD was given (2,000 units), and the rings were again constricted with norepinephrine. Once the norepinephrine constriction had plateaued, A23187 (10^-6 M) was given. Nitric oxide levels were drawn before and after the administration of SOD and A23187.

Endothelial Culture

Bovine PA endothelial cells were obtained commercially (American Type Culture Collection, Bos taurus line No. CCL209) and grown to confluence on customized culture plates using standard growth media. The cells were studied between the 16th and 30th passages in culture. The floor of each plate contained a central “cutout” sealed by a coverslip. The coverslip was fastened in place by a 1:1:1 mixture of bee’s wax, petroleum jelly, and paraffin. When the cells were confluent, they were loaded with fura 2, as described below, and the entire plate was placed on the heated stage (37°C) of an epifluorescence microscope (Nikon Diaphot, Nikon Instrument Group Inc., Garden City, N.Y.), which permitted Ca²⁺ measurement from five to ten visually selected cells.

Fura Loading

Measurement of cytosolic calcium was performed using modifications of the methods described by Wickham et al.²⁵ Plates were washed three times with Hanks’ buffered saline solution (HBSS) and then incubated with 10 μM fura 2-AM (Molecular Probes, Inc., Eugene, Ore.) at 37°C for 40 minutes. The cells were again washed three times with HBSS, and then the plates were incubated at 37°C for 20 minutes to allow cleavage of the acetoxymethyl ester. The plates were flushed three times with HBSS (buffered with HEPES buffer to pH 7.4) to remove uncleaved fura 2-AM.

Fluorescence Microscopy

Cells on the coverslip portion of the plate were viewed using a ×40 oil/fluor objective. The microscope was coupled to a dual-channel spectrofluorometer (L-format, model AR-CM, SPEX Industries, Inc., Edison, N.J.), which stimulated the cells at 340 and 380 nm with a 150-W, ozone-free xenon lamp. The 340- and 380-nm stimulations were performed at 950 and 500 V, respectively. Neutral density filters (Corion Corp., Holliston, Mass.) kept counts in the unstimulated state ≤1×10^5 cfs, avoiding saturation of the photomultiplier tube. Before each experiment, the reference values for the 340- and 380-nm signals were checked and were maintained between 1.0 and 0.5. Cell stimulation was accomplished by a computerized, filter-based system that rapidly alternated between stimulating wavelengths (340 and 380 nm) using a chopper. Intracellular, free cytosolic calcium

Materials and Methods

Pulmonary Artery Ring Model

Adult, male, specific pathogen-free Sprague Dawley rats were anesthetized (50 mg/kg i.p. nembutal) and placed on a warming table. The rats were ventilated with humidified room air via a tracheotomy; a small animal ventilator (70 breaths/min, 3 ml tidal volume, 3 cm H₂O positive end-expiratory pressure, Harvard Apparatus, South Natick, Mass.) was used. The lungs were exposed via thoracotomy, as previously described.²³ The second division of the right PA was dissected free with iris scissors, avoiding endothelial injury. In some experiments, the endothelium was removed by gentle rubbing with a metal probe. Scanning electron micrographs of representative rings were obtained to document the efficacy of this technique (Figure 5). Rings were mounted on stainless-steel wires attached to force-displacement transducers (model UC-2, Grass Instrument Co., Quincy, Mass.) and suspended in a 75-ml bath containing Earle’s solution (pH 7.430±0.001; temperature, 37.5±0.1°C; PO₂, 130–140 mm Hg). The rings (two rings per bath) were held 1 cm apart, and nitric oxide samples were withdrawn from midway between vessels using a 1-ml gas-tight syringe. Rings were allowed to equilibrate for 30 minutes and then maintained at a resting tension of 800 mg.

After equilibration, each vessel was constricted with norepinephrine (3.1×10^-8 M). Once the constriction had plateaued, the rings were given either BK (10^-8-10^-6 M, n=12), ACh (10^-6-10^-5 M, n=10), or A23187 (10^-6 M, n=12). All drugs were obtained from Sigma Chemical Co., St. Louis, unless stated otherwise. Vessel tension was monitored continuously. Nitric oxide samples were drawn before and after administration of norepinephrine and each of the three vasodilators (Figures 2–4). The bath was then flushed clean. Once PA tension had reequilibrated, superoxide dismutase (SOD) was given (2,000 units, bovine SOD, Sigma), and after 10 minutes, rings were constricted with norepinephrine. When the norepinephrine constriction had plateaued, the rings were exposed to the same doses of the same dilator they had received before SOD administration. Nitric oxide samples were drawn before and after administration of SOD, norepinephrine, and each of the three vasodilators (Figures 2–4). SOD was used to prevent destruction of nitric oxide by superoxide anion, in hopes of prolonging the survival of nitric oxide.

The effects of meclofenamate (9×10^-5 M) pretreatment on ACh-, BK-, and A23187-induced vasodilation was assessed in an additional five rings. Vasodilator responses to ACh were compared in 10 additional rings before and after ouabain (10^-5–10^-3 M).

Nitric oxide,²⁴ the current study examined the effects of three endothelium-dependent vasodilators (ACh, BK, and A23187) on these variables in cultured pulmonary artery (PA) endothelial cells and rat PA rings.
NITRIC OXIDE PRODUCTION AND PULMONARY VASODILATION

**Figure 1.** Bar graph showing calibration of the nitric oxide assay in vitro. Chemiluminescence increases in proportion to nitric oxide concentration. Values are mean ± SEM; n = 30. The chemiluminescence caused by a known dose of gaseous (hatched bars) or dissolved (closed circles) nitric oxide was plotted on the y axis in millivolts. Integration time was 0.01 seconds. †Chemiluminescence produced by a given dose of nitric oxide (gas or saturated liquid) differs from level produced by all other doses at p < 0.05.

<table>
<thead>
<tr>
<th>Nitric Oxide (pmol)</th>
<th>Chemiluminescence (mV)</th>
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<tbody>
<tr>
<td>10.0 pmol</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>20.0 pmol</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>30.0 pmol</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>40.0 pmol</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>50.0 pmol</td>
<td>2.5 ± 0.5</td>
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**Figure 2.** Graphs showing that acetylcholine (ACH) causes vasodilation of pulmonary artery (PA) rings but does not result in nitric oxide synthesis. This figure is an artist's representation of a typical experiment. The values on the figure are mean ± SEM of 10 experiments. Upper left panel: \(10^{-7} + 10^{-6} + 10^{-5} \text{M} \) ACH causes vasodilation of norepinephrine (NE)-constricted PA rings. Lower left panel: Nitric oxide was not detected in samples drawn within 30 seconds of ACH administration. Upper right panel: ACH-induced vasodilation of NE-constricted PA rings was enhanced by superoxide dismutase (SOD). Lower right panel: There was no nitric oxide signal after administration of ACH, despite the administration of SOD. *Timing of the nitric oxide sample (indicated on the lower panel) relative to the PA ring tension (noted on the corresponding upper panel). \( \Delta T_{ACH} \) (the change in tension achieved by acetylcholine/plateau tension before acetylcholine) \( \times 100 \).

Chemiluminescence produced by all gaseous nitric oxide (gas standard) was 18.0 mV while chemiluminescence produced by all dissolved nitric oxide (saturated liquid) was 16.0 mV. Differences between the two chemiluminescence levels was significant (p < 0.05). The chemiluminescence caused by a known dose of nitric oxide was plotted on the y axis in millivolts. Integration time was 0.01 seconds. The chemiluminescence produced by a given dose of nitric oxide differs from level produced by all other doses at p < 0.05.

Chemical Production and Pulmonary Vasodilatation

Concentration ([Ca\(^{2+}\)]) was calculated using the equation of Grynkiewicz et al.\(^{26}\):

\[
[Ca^{2+}] = K_d \cdot \frac{(R-R_{min})}{(R_{max}-R)} \cdot \frac{F_{min,380}}{F_{max,380}}
\]

where \(K_d\) is the dissociation constant for intracellular calcium, \(R_{max}\) and \(R_{min}\) are the ratios obtained by lysing the cells and saturating the fura 2 with calcium and then chelating all free calcium with EGTA, and \(F_{min,380}\) and \(F_{max,380}\) are the fluorescence intensities of fura 2 in the free and bound states at 380 nm, respectively. \(R_{max}\) and \(R_{min}\) were determined on each plate at the end of the experiment. Experiments were conducted within 5 minutes of fura loading and were performed in the dark to optimize the signal/noise ratio.

**Determination of Maximum and Minimum Values**

\(R_{max}\) and \(R_{min}\) calcium values were determined for each plate of cells, and plates in which the \(R_{max}\) did not exceed the agonist-stimulated signal were discarded, because this indicated inadequate fura load-
ing. The maximum Ca\(^{2+}\) value was determined by permeabilizing the membrane with ionomycin (10 \(\mu\)mol/l) and then lysing the cells with the detergent Triton X-100 (20 \(\mu\l\) of a 1/100 dilution of stock, Sigma). EGTA (2 mmol/l) was then given to bind all Ca\(^{2+}\) creating the “minimum Ca\(^{2+}\) state.” Ionomycin consistently produced a lower \(R_{\text{max}}\) than Triton X-100, and so the \(R_{\text{max}}\) caused by Triton X-100 was used for calculation of free cytosolic calcium concentration. The dose of Triton used did not cause significant autofluorescence.

**Measurement of Cytosolic Calcium \(n=78\)**

ACh (10\(^{-8}\) M), A23187 (10\(^{-8}\) M), and BK (10\(^{-8}\) M) were studied after administration of 1 mmol CaCl\(_2\) \((n=10\) each). ACh and A23187 were also studied at higher doses (10\(^{-6}\) M, \(n=5\) experiments each). In 12 additional experiments, Ca\(^{2+}\)-replete cells were given atropine (1.43\(\times\)10\(^{-6}\) M) before challenge with ACh (10\(^{-8}\) M). BK, ACh, and A23187 were given 5 minutes after administration of 1 mmol Ca\(^{2+}\), at which time the fluorescence signal had achieved a new, stable level. Once the response to the study drug was complete, \(R_{\text{max}}\) and \(R_{\text{min}}\) were measured, and calcium concentrations were calculated using the computer software. ACh, A23187, and BK were also given to cells in calcium-free media \((n=5\) each).

**Nitric Oxide Measurement**

Nitric oxide was measured using a nitric oxide analyzer (Sievers Research Inc., Boulder, Colo.). Detection of nitric oxide is based on the observation that ozone \((O_3)\) plus nitric oxide \((NO)\) emits light \((hv)\): NO+O\(_3\)->O\(_2\)+NO\(_2\)+hv. Perfusate from PA rings was injected into a glass purge chamber that was evacuated to a pressure of 3 mm Hg by a vacuum pump before sample injection. Nitric oxide was “stripped” from the liquid to the gas phase by bubbling it with nitrogen \((12\ \text{ml/min})\) for 1 minute. The gaseous portion of the specimen was drawn into a chamber situated in front of a cooled, red-sensitive, photon-counting tube (Hamamatsu Corp., Bridgewater, N.J.). Here it mixed with \(O_3\), which was generated internally by electrostatic discharge. The nitric oxide analyzer was calibrated by measuring the chemiluminescence signal produced by known amounts of gaseous nitric oxide \((10.0, 20.3, 30.8, 41.0, \text{and } 82.0\ \text{pmol}; n=5\) measurements/dose) or an aqueous solution of nitric oxide in
saline (40 and 80 ppm; n=4 measurements/dose). Nitric oxide solutions were prepared using double-distilled water that had previously been bubbled with helium for 30 minutes to remove O₂. The water was then bubbled with 100% nitric oxide for 30 minutes and placed in a 125-ml air-tight bomb. Nitric oxide dose calculation was based on a molecular weight of 30.006 (1 mol NO=0.030006 kg) and an absolute density of 101.325 kPa at 25°C with corrections for ambient barometric pressure and room temperature. The nitric oxide tanks were handled by a technician wearing a protective mask and operating in an externally vented fume hood.

Endothelial Cells on Beads

Endothelial cell culture on microcarrier beads was performed as previously reported by Ryan et al.27 Cytodex 3 microcarrier beads (Pharmacia LKB Bio-

![Graphs showing that bradykinin (BK) causes vasodilatation and nitric oxide synthesis in pulmonary artery (PA) rings.](image)

**FIGURE 4.** Graphs showing that bradykinin (BK) causes vasodilatation and nitric oxide synthesis in pulmonary artery (PA) rings. This figure is an artist’s representation of a typical experiment. The values on the figure are mean±SEM of 12 experiments. Upper left panel: BK causes minimal vasodilatation of norepinephrine (NE)-constricted PA rings. Lower left panel: Nitric oxide is not detected in samples drawn within 30 seconds of BK administration. Upper right panel: BK-induced vasodilatation of NE-constricted PA rings is not enhanced by superoxide dismutase (SOD). Lower right panel: A nitric oxide signal is evident after administration of BK to SOD-pretreated PA rings. There is no nitric oxide signal before the administration of BK. *Timing of the nitric oxide sample (indicated on the lower panel) relative to the PA ring tension (noted on the corresponding upper panel). ΔT<sub>BK</sub>, the reduction in PA tension achieved by BK/plateau tension before BK) ×100.

### Table 1. Meclofenamate Reduced Acetylcholine-Induced Pulmonary Vasodilatation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ACh (10⁻⁷–10⁻⁶ M)</th>
<th>A23187 (10⁻⁷ M)</th>
<th>BK (10⁻⁸–10⁻⁶ M)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ΔTension mg ± %</td>
<td>ΔTension mg ± %</td>
<td>ΔTension mg ± %</td>
</tr>
<tr>
<td>Control</td>
<td>−552±60 −56±4</td>
<td>−153±38 −11.5±2.7</td>
<td>−45±16 −11±3</td>
</tr>
<tr>
<td>Ouabain</td>
<td>−559±31 60±2</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Meclofenamate (9×10⁻³ M)</td>
<td>−248±23* −22.1±1.9</td>
<td>−138±20.6 −11.5±1.7</td>
<td>−80±33 −7.1±2.7</td>
</tr>
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</table>

Values are mean±SEM. ΔTension, change in tension in pulmonary artery rings caused by each endothelium-dependent vasodilator (EDV) before or after meclofenamate or ouabain; ACh, acetylcholine; A23187, an endothelium-dependent vasodilator; BK, bradykinin. The percent change in tension (T) caused by each dilator was calculated as: T<sub>post EDV</sub>−T<sub>pre EDV</sub>/T<sub>pre EDV</sub>×100.

*p<0.05 vs. control.
technology, Piscataway, N.J.) were prepared by soaking 1 g dry beads in 100 ml phosphate buffered solution (Mg$^{2+}$ and Ca$^{2+}$ free) for 3 hours at room temperature. The beads were then rinsed with phosphate buffered solution and autoclaved (121°C and 21 psi for 3 hours). Beads were placed on confluent plates of bovine PA endothelial cells. The cells grew onto the beads, permitting harvest of cell-covered beads in 7 days. Beads were harvested by briskly tapping the bottom of the flask to dislodge them. Then they were washed with HBSS solution to remove culture media. The beads were then allowed to sediment passively. The precipitated beads (1.5 ml) were suspended in HBSS solution in a 5-ml plastic syringe. The analyzer was readied, and then the test agent was added to the syringe in the presence of SOD (2,000

units). The syringe was shaken for 30 seconds, and then the fluid was injected into the vacuum chamber of the nitric oxide analyzer through glass wool and a 0.2-μm filter (Nuclepore Corp., Pleasanton, Calif.). Twenty-four experiments were performed (A23187 alone, n=12; A23187+2,000 units SOD, n=4; 10⁻⁶ M ACh and 10⁻⁶ M BK, n=4 each).

Statistics

Values were given as mean±SEM. Analysis of variance was used for comparison among three or more groups. Post hoc analysis was performed using a Fisher least significant difference test. Comparison between two groups was performed with a two-tailed
was

3). ACh (Figure

-51±4% 

concentration.

4). ACh-induced constriction from 407±27 to 788±38 mg (p<0.05) but did not reduce the subsequent ACh-induced vasodilatation (Table 1). Neither ACh nor A23187 caused vasodilatation of denuded PA rings. Scanning electron micrographs comparing luminal surfaces in denuded versus intact rings confirmed that the denuded rings were devoid of endothelial lining (Figure 5). There was also no nitric oxide production by the denuded rings at baseline or in response to A23187 (10⁻⁶ M), ΔTₐ₂₃₁₈ (the reduction in PA ring tension achieved by A23187/placebo tension before A23187) ×100.

Nitric Oxide Production by Pulmonary Artery Rings and Endothelial Cells

There was no basal nitric oxide production detected (Figures 2–4). Only A23187 consistently caused detectable nitric oxide production in the absence of SOD (Figure 3). The magnitude of the A23187-induced nitric oxide signal was greatly enhanced by SOD pretreatment (Figure 3). BK also caused a small, but consistent, nitric oxide signal when rings were pretreated with SOD. ACh failed to cause detectable nitric oxide production even in the presence of SOD.

Without SOD, only two of 12 attempts to detect nitric oxide production from A23187-stimulated beads were successful, whereas two of four experiments in which beads were pretreated with SOD resulted in a signal of >1–2 mV. ACh and BK failed to cause a detectable nitric oxide signal when administered to the endothelial cell–coated beads.

Cytosolic Calcium

The administration of physiological levels of calcium to the cultured PA endothelial cells (which had been in calcium-free media during the fura loading) resulted in a rapid rise in cytosolic calcium, followed by a decline, over 5 minutes, to a stable plateau level (Figure 6).

Student’s t test. A value of p<0.05 was considered statistically significant.

Results

Nitric Oxide Analyzer Calibration

Nitric oxide gas was consistently detected in amounts as low as 10 pmol, but there was not a statistical difference between the signal caused by 10 and 20 pmol. Each increment in the nitric oxide dose from 30 to 80 pmol caused a significant increase in the nitric oxide chemiluminescence signal (p<0.01, Figure 1). Nitric oxide given in a saline solution at 40- and 80-pmol doses caused a chemiluminescence signal that was similar in magnitude to that produced by an equivalent dose of gaseous nitric oxide (Figure 1). This indicated that the stripping of nitric oxide from solution was effective in mobilizing dissolved nitric oxide into the gaseous phase (Figure 1). Nitrogen gas, solutions of sodium nitrite (1 mM), and potassium nitrate (1 mM) failed to cause any chemiluminescence signal.

Pulmonary Artery Rings

ACh caused the most vasodilation of the three drugs (Figure 2). A23187 resulted in an intermediate degree of dilation (Figure 3), whereas BK caused only minor vasodilation of norepinephrine-constricted PA rings (Figure 4).

SOD did not alter the vasodilation caused by A23187 (−12±3% both before and after SOD) (Figure 3). Neither did it enhance BK-induced vasodilatation (−11±3% before SOD and 6±2% after SOD) (Figure 4). ACh not only caused a greater degree of pulmonary vasodilation than did BK or A23187 (−51±4% before SOD, p<0.01), but this vasodilation was enhanced by SOD (−94±4% vasodilation after SOD; p<0.01 value differs from BK or A23187 group) (Figure 2).

The magnitude of the norepinephrine-induced constrictions was not altered by SOD and was similar among the three groups (Figures 2–4). Perfusate pH/Po₂ was 7.40±0.02/144±3 mm Hg in the ACh experiments, 7.42±0.01/145±1 in the A23187 experiments, and 7.42±0.01/143±1 mm Hg in the BK experiments. Rat weight was not different among ACh, A23187, and BK groups (278±5, 276±5, and 279±9 g, respectively). The rings were also similar in size (length×width) among ACh, A23187, and BK groups (3.00±0.06×2.48±0.01, 3.05±0.04×2.50±0.00, and 2.97±0.04×2.49±0.01 mm, respectively).

Resting PA ring tension was not altered by meclofenamate (0.0±0.0 mg) nor ouabain (−19±6 mg). Meclofenamate reduced ACh-induced vasodilation (p<0.05) but did not alter the effects of BK or A23187 (Table 1). Ouabain enhanced norepinephrine-induced constriction from 407±27 to 788±38 mg (p<0.05) but did not reduce the subsequent ACh-induced vasodilatation (Table 1). Neither ACh nor A23187 caused vasodilatation of denuded PA rings. Scanning electron micrographs comparing luminal surfaces in denuded versus intact rings confirmed that the denuded rings were devoid of endothelial lining (Figure 5). There was also no nitric oxide production by the denuded rings at baseline or in response to A23187 (10⁻⁶ M), ΔTₐ₂₃₁₈ (the reduction in PA ring tension achieved by A23187/placebo tension before A23187) ×100.

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Calcium-Replete Media

The effect of ACh on cytosolic calcium was dose dependent. The changes in cytosolic calcium concentration caused by $10^{-8}$, $10^{-7}$, $10^{-6}$, and $10^{-5}$ M ACh were $-15 \pm 3.8$, $+58 \pm 45$, $+2.9 \pm 7.6$, and $-17.7 \pm 14.2$ nM, respectively. The heterogeneity of the response to ACh related to the fact that, although ACh modestly lowered cytosolic calcium in most experiments at most doses (Figure 7), the higher doses occasionally resulted in marked elevation of cytosolic calcium levels. Atropine lowered cytosolic calcium and reduced the fall in cytosolic calcium concentration caused by ACh (Figure 8). A23187 and BK both increased the free cytosolic calcium concentration (Figures 7 and 9).

Calcium-Free Media

BK increased the free cytosolic calcium concentration in cells studied in a calcium-free environment. Although the increase in free cytosolic calcium concentration was qualitatively similar to that observed in cells studied in the presence of 1 mM Ca$^{2+}$, the absolute free cytosolic calcium concentration achieved in the calcium-deficient media was less (Figure 9). ACh tended to increase intracellular calcium concentration in calcium-free media (Figure 9). A23187 ($10^{-8}$ M) increased free cytosolic calcium concentration in cells studied in the calcium-free state to a similar extent as when the drug was given in the presence of extracellular calcium (Figure 9).

Discussion

Endothelium-dependent pulmonary vasodilatation caused by A23187 and BK was associated with nitric oxide synthesis (Figures 2–4) and elevation of PA endothelial cytosolic calcium concentration (Figure 7). Computer-scanned images showing that bradykinin increases and acetylcholine reduces the cytosolic calcium concentration in pulmonary artery endothelial cells. These images are from actual experiments and are representative of typical experiments. The cells were first given 1 mM Ca$^{2+}$. Once the signal had stabilized, they received bradykinin ($10^{-8}$ M, top panel) or acetylcholine ($10^{-8}$ M, bottom panel). The maximum and minimum calcium values were then obtained by administration of ionomycin/Triton X-100 followed by EGTA (as described in the text).
9). In contrast, the endothelium-dependent relaxation caused by ACh resulted in no generation of nitric oxide and in reduced endothelial cytosolic calcium concentration (Figures 7 and 8). ACh was the prototype endothelium-dependent vasodilator and has often been assumed to act by stimulating EDRF synthesis or, more recently, via the nitric oxide pathway. However, there are considerable data that refute this contention. For example, in the rat lung, LNMMA, a putative inhibitor of nitric oxide synthesis, blocks the effects of BK but not ACh.2 In the dog coronary artery, ACh- but not BK-induced vasodilatation is blocked by ouabain, an inhibitor of the Na+,K+-ATPase pump.10 Menon et al.21 provided the first direct evidence that ACh did not cause nitric oxide synthesis in the pulmonary vasculature. They reported that A23187, but not ACh, caused nitric oxide production from bovine pulmonary arteries. Since the endothelium can mediate pulmonary vasodilatation by several nitric oxide-independent mechanisms, including production of prostanoids and EDHF,7,8 correlation of nitric oxide production with vasodilation is essential to differentiate among mechanisms.

**A23187 and BK, but Not ACh, Stimulated Nitric Oxide Synthesis**

In the current study, nitric oxide became detectable 15–30 seconds after administration of A23187 or BK. This nitric oxide was proven to originate in the endothelium, since A23187 administration did not stimulate nitric oxide synthesis when given to denuded rings, even in the presence of SOD. Denuded rings displayed a loss of the typical “cobblesstone” appearance of the luminal surface on scanning electron microscopy (Figure 5). They also failed to dilate in response to ACh.

The few direct demonstrations that ACh can elicit nitric oxide synthesis have been performed in systemic vessels and used chemiluminescence assays.
that acidified the specimen in the presence of potassium iodide.\textsuperscript{14,15,17} The failure of ACh to elicit nitric oxide synthesis in the lung could be due to differences between pulmonary and systemic vessels; certainly the pulmonary vasculature differs from most systemic circulations in its response to hypoxia (constriction instead of dilatation). Alternatively, apparent differences in the effects of ACh on nitric oxide synthesis between the circulations might result from the types of chemiluminescence assays used. The absence of the acidification step in the current assay (made possible by the exclusion of O\textsubscript{2} from the assay) greatly enhances the specificity of the technique, largely by preventing nitrosocompounds, nitrates, and nitrates from being converted to nitric oxide during measurement.\textsuperscript{28} The current study and that of Menon et al\textsuperscript{21} are the only two that have evaluated the effects of ACh on nitric oxide production in the pulmonary circulation using a nonacidified chemiluminescence technique; both failed to detect ACh-induced nitric oxide synthesis.

ACh-induced vasodilatation was partially inhibited by the cyclooxygenase blocker meclofenamate. This presumably represents the inhibition of production of ACh-induced prostacyclin synthesis. Meclofenamate, at doses that significantly inhibited ACh-induced prostaglandin synthesis, failed to prevent the early and preponderant dilator response to ACh in the isolated rat lung.\textsuperscript{3,29} It is unlikely that ACh-induced vasodilatation resulted from nitric oxide production at levels below the detection threshold of the current assay, since the much weaker vasodilatation elicited by BK and A23187 caused measurable nitric oxide synthesis. The fact that ACh was a more potent pulmonary vasodilator than either BK or A23187 suggests that the mechanism used by ACh may be a more important determinant of pulmonary vascular tone than the nitric oxide pathway. The magnitude of vasodilatation caused by ACh could not be achieved by giving higher doses of A23187 or BK; in fact, this strategy resulted in vasoconstriction. If ACh-induced vasodilatation was not the result of nitric oxide synthesis or prostaglandin generation, what was its mechanism?

The most probable mechanism for ACh-induced vasodilatation is membrane hyperpolarization. Chen and Suzuki\textsuperscript{30} recently demonstrated that ACh caused an endothelium-dependent hyperpolarization of rabbit carotid arteries, which was inhibited by procaine, an agent that depolarizes smooth muscle and inhibits mobilization of intracellular calcium. They noted a transient and a sustained phase to the ACh-induced hyperpolarization, only the latter of which was dependent on extracellular calcium concentration. Since ouabain has been reported to inhibit ACh-induced dilatation in systemic canine arteries,\textsuperscript{31} possibly by acting as an inhibitor of EDHF, rings were pretreated with ouabain. However, large doses of ouabain (up to 10\textsuperscript{-3} M) enhanced norepinephrine-induced constriction without reducing ACh-induced vasodilatation (Table 1). Verification that ACh causes pulmonary vasodilatation by producing EDHF (or otherwise hyperpolarizing the vascular smooth muscle) will require direct measurement of membrane potential and assessment of the effects of the ACh-PA endothelial cell interaction on the activity of smooth muscle K\textsuperscript{+} channels via patch-clamping.

\textbf{SOD Enhanced Detection of Nitric Oxide}

SOD enhanced the detection of nitric oxide. This is consistent with previous bioassay studies in which superoxide anion (\cdot O\textsubscript{2}\textsuperscript{-}) shortened\textsuperscript{32} and SOD prolonged \textsuperscript{1/2} of EDRF.\textsuperscript{33} SOD increased the size of the chemiluminescence signal in response to A23187, the most potent of the nitric oxide--producing agents. However, a signal was usually detectable even in the absence of SOD (Figure 3). This presumably reflects the ability of SOD to scavenge radicals produced by the PA ring, thus preventing these radicals from destroying nitric oxide. Previous studies\textsuperscript{34,35} have documented the physiological production of O\textsubscript{2} radicals in the normoxic rat lung. BK only elicited detectable nitric oxide when rings had been pretreated with SOD (Figure 4). This may reflect the ability of BK to stimulate radical production by increasing cyclooxygenase metabolism.\textsuperscript{12} If BK administration increased radical production, SOD might be required to stabilize the nitric oxide long enough to permit its diffusion into the perfusate, which is essential for its detection. ACh did not lead to nitric oxide production even in the presence of SOD, and yet ACh-induced pulmonary vasodilatation was enhanced by SOD (Figure 2). This suggests that EDHF, or another intermediary in the as-yet-undefined ACh vasodilation sequence, is sensitive to \cdot O\textsubscript{2}\textsuperscript{-} and, like nitric oxide, is protected by the administration of SOD.

It is intriguing that SOD increased the magnitude of the nitric oxide signal produced in the perfusate around the PA rings by A23187 and BK and yet failed to enhance vasodilatation. This could mean that nitric oxide is only a marker for the "true" EDRF, which could be a related compound, such as a nitrothiol. Alternatively, the amount of nitric oxide that diffuses into the bath might not reflect the concentration of nitric oxide at the endothelial cell--smooth muscle junction. Since SOD is a large molecule and unable to enter the vascular wall rapidly, it may only protect nitric oxide that "leaks" from the endothelial cell. This would leave the more important "pool" of nitric oxide that is in direct contact with the smooth muscle cell susceptible to destruction by radicals. There was no basal release of nitric oxide detected even in the presence of SOD. This may reflect the true absence of basal production or indicate that the level of nitric oxide production in these small rings does not exceed the threshold for detection of this assay (10 pmol) within the diffusion radius measured, \textasciitilde1 cm.

\textbf{Endothelium-Dependent Dilators, Which Caused Nitric Oxide Synthesis (A23187 and BK), Elevated Cytosolic Calcium}

A common feature of endothelium-dependent vasodilators is their propensity to elevate cytosolic
calcium.\textsuperscript{24,32,36,37} Furthermore, although EDRF activity can occur in Ca\textsuperscript{2+}-free media, its magnitude is reduced, and a sustained response requires adequate extracellular Ca\textsuperscript{2+}.\textsuperscript{23,36,38} Few studies have correlated the effects of endothelium-dependent vasodilators on free cytosolic calcium concentration with their production of EDRF bioactivity. Peach et al.\textsuperscript{36} demonstrated that doses of BK that caused maximal elevation of cytosolic Ca\textsuperscript{2+} also caused the optimal EDRF activity, as measured by bioassay.

In the current study, the time to onset of nitric oxide appearance (15–30 seconds) was delayed relative to the time course of the rise in cytosolic calcium (<5 seconds) observed in PA endothelial cells treated with the same doses of the same dilators. Although endothelial calcium concentration and nitric oxide production could not be measured simultaneously in the same tissue, this temporal sequence is consistent with the hypothesis that nitric oxide synthesis is initiated by an endothelium-dependent dilator causing a rise in endothelial cell cytosolic calcium (Figures 7 and 9). Although calcium-replete cells had higher resting and BK-stimulated cytosolic calcium concentrations than cells studied in the absence of extracellular calcium, the dilator-induced change in cytosolic calcium was not different in calcium-free versus calcium-replete cells (Figure 9). This suggests the release of intracellular calcium is important in the BK response. The current demonstration that BK (10\textsuperscript{-8} M) increased free cytosolic calcium concentration from 55.9±7.8 to 96.6±18.1 nM is consistent with that of Sage et al.\textsuperscript{39}

In contrast, ACh reduced cytosolic calcium (Figures 7 and 8). The failure of free cytosolic calcium concentration to increase in response to most doses of ACh was not due to the absence of muscarinic receptors, since the effect of ACh was largely inhibited by atropine (Figure 8). Furthermore, cytosolic calcium tended to increase in response to ACh in cells studied in calcium-free media (Figure 9). The mechanism by which ACh lowers cytosolic calcium was not addressed in the current study but could relate to membrane hyperpolarization, activation of Ca-ATPase or stimulation of the Na-Ca exchanger. To establish that the cytosolic calcium measurements were made in cells with an intact nitric oxide pathway, each of the vasodilator drugs was given to a column of microcarrier beads coated with PA endothelial cells. These cells did produce nitric oxide (≤20 pmol) in response to A23187, but not to ACh. The dissimilarity of the effects of ACh versus BK and A23187 on PA endothelial cytosolic calcium concentration provides another piece of evidence that these drugs do not share a common mechanism of pulmonary vasodilatation.

Conclusions

This study demonstrates that the pulmonary vasodilatation caused by BK and A23187 is associated with rapid elevation of endothelial cell cytosolic calcium concentration and nitric oxide synthesis, whereas the dilatation caused by acetylcholine neither stimulates nitric oxide synthesis nor elevates cytosolic calcium concentration. The mechanism of ACh-induced pulmonary vasodilatation is unknown but has been shown to be endothelium dependent and to persist in the presence of ouabain and meclofenamate.

References

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from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 1987;84:9265–9269


**KEY WORDS** • nitric oxide • pulmonary vascular reactivity • endothelium-derived relaxing factor • acetylcholine • cytosolic calcium
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