Close Correlation between Cytoplasmic Ca^2+ Levels and Release of an Endothelium-Derived Relaxing Factor from Cultured Endothelial Cells

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ABSTRACT. We studied whether there is a quantitative relationship between free cytosolic Ca^2+ levels and the release of an endothelium-derived relaxing factor (EDRF) from cultured fetal bovine aortic endothelial cells (EC). EC pretreated with indomethacin were stimulated by the agonists adenosine triphosphate (ATP), bradykinin (BKN), acetylcholine (ACh) and calcium ionophore (A23187) in various concentrations (10^-8-10^-5 M), and the amount of EDRF released was determined on the basis of endothelium-free rabbit aortic ring relaxation and cultured smooth muscle cell cGMP content. Changes in intracellular Ca^2+ concentration ([Ca^2+]_i) in response to the same stimuli were determined by photometric fluorescence microscopy using the fluorescent calcium indicator Fura-2. EC stimulation by ATP and A23187 induced dose-dependent increases in both [Ca^2+]_i and the amount of EDRF released. BKN increased both [Ca^2+]_i and EDRF release upon initial exposure (10^-8 M), but there were no further changes at higher concentrations. ACh induced no significant changes in either [Ca^2+]_i or EDRF release. There was a close quantitative correlation between agonist-induced changes in [Ca^2+]_i and the amount of EDRF released (relaxation response in aortic rings and cGMP levels.) (p < 0.001) Removal of extracellular Ca^2+ eliminated continuous elevation in both [Ca^2+]_i and the amount of EDRF induced by ATP (10^-5 M), BKN (10^-8 M) and A23187 (10^-6 M). These findings suggest that intracellular Ca^2+ levels are directly linked to the amount of EDRF released, and that extracellular Ca^2+ is essential for EDRF release because its influx is involved in the continuous elevation of [Ca^2+]_i.

Vascular endothelial cells produce and secrete vasodilator and vasoconstrictor substances which are closely related to regulation of vascular tonus (2, 8, 12, 32, 36). Endothelium-derived relaxing factor (EDRF) (11), a vasodilator substance produced by endothelium, is believed to be nitric oxide (NO) (27, 28) or an NO-related substance such as S-nitroso-L-cysteine (25). EDRF activates the intracellular soluble guanylate cyclase of subendothelial vascular smooth muscle and elevates cGMP to induce dephosphorylation of myosin light chain, leading to smooth muscle relaxation (24, 30). EDRF release from the endothelium is stimulated by hormones or autacoids in the blood stream, or by neurotransmitters. It is known to be affected not only by such chemical agonists but by physical stimulation such as shear stress caused by blood flow (10, 22, 31). The details of the mechanism of EDRF release in response to the chemical or physical stimulation of endothelial cells have been progressively elucidated.

Intracellular free Ca^2+ has the role of second messenger by which information on exogenous stimuli is transmitted to the interior of many types of cells, and changes in its concentrations are related the cell's response to stimulation, i.e., changes in cell functions (3, 7). Ca^2+ has recently been shown to participate in the release of EDRF caused by agonists (1, 13, 14, 16), and more recently it has been shown that cytosolic free Ca^2+ directly affects the activity of the EDRF-forming enzyme (NO synthase) present in the cytosol of endothelial cells (21). However, there is little information concerning the quantitative relationship between intracellular free Ca^2+ concentration and the release of EDRF from vascular endothelial cells.

The present study was therefore undertaken to investigate quantitatively the relationship between changes in intracellular Ca^2+ concentration and the amount of EDRF released upon stimulation with agonists. Cul-
tured fetal bovine descending aorta endothelial cells were stimulated by the agonists adenosine triphosphate (ATP), acetylcholine (ACh), bradykinin (BKN) and calcium ionophore (A23187), and the amount of EDRF released was determined by bioassay using specimens of rabbit aorta and radioimmunoassay of cyclic guanosine-3',5'-monophosphate (cGMP) in cultured smooth muscle cells. Changes in intracellular Ca\(^{++}\) concentration ([Ca\(^{++}\)]) due to the same stimuli were also determined by photometric fluorescence microscopy using Fura-2, a fluorescent calcium indicator.

**MATERIALS AND METHODS**

**Cell culture.** Endothelial cells were isolated from the bovine fetal descending aorta by collagenase (0.1%) digestion and cultured in M199 medium containing 20% fetal bovine serum, 100 U penicillin per ml and 100 μg streptomycin per ml at 37°C in an atmosphere of 95% air and 5% CO\(_2\). Whenever the cells became confluent, they were separated with a 0.05% Trypsin-2 mM EDTA solution and subcultured at a density of 1 x 10\(^4\) cells/cm\(^2\) in a new flask. In each subculture the cells were counted with a Coulter counter, and the number of cumulative population doublings (CPD) was calculated. All the cells used in this study had fewer than 30 CPDs. The cultured cells were confirmed to be endothelial cells by the morphological findings, i.e., a single layer pavement-like arrangement characteristic of endothelial cells, the presence of factor VIII-related antigen revealed by the fluorescent antibody technique, the production of angiotensin-I converting enzyme, and the uptake of acetylated low-density lipoproteins. Endothelial cells were cultured on a glass coverslip (0.05 x2x4 cm), and confluent cells were used for [Ca\(^{++}\); ] determinations. Cells with the same CPDs as that of cells subcultured on the same day were used for the experiment on EDRF and the determination of Ca\(^{++}\) concentration.

In the experiment on EDRF, cells were added to microcarrier beads (Cytodex 3, beads ϕ=175 μm) at a density of 6.6 x 10\(^5\) cells/ml of beads in a gyratory culture bottle (Technie, Princeton, U.S.A) and cultured. A stirrer in the bottle was rotated in cycles of 50 rpm for 2 min every 30 minutes during the initial 5-hour period, for cells to attach to the beads. The stirrer was subsequently rotated at 50 rpm, and the cells were cultured in suspension until they became confluent (about 3-4 days).

Smooth muscle cells were primarily cultured by the explant method after tissue sections (2 mm x 2 mm) of tunica media were isolated from blood vessels whose endothelial cells had been removed by the method described above. M199 medium supplemented with 10% fetal bovine serum was used. Subculture was repeated every week after the primary culture, and cells with fewer than 30 CPDs were distributed in a 6-well plate (FALCON). The cultured cells were confirmed to be smooth muscle cells by their morphology (spindle shape and the "hill and valley" pattern as a result of multilaminar growth) and the results of anti-α-isoactin antibody (Sigma Chemical Co. St. Louis) staining.

**Determination of intracellular Ca\(^{++}\) concentration.** [Ca\(^{++}\)]\(_i\) was determined with Fura-2, a fluorescent Ca\(^{++}\) indicator, and a photometric fluorescence microscope (NIKON MICRO-PHOTO FX-P1). The cultured endothelial cells on the coverslip were incubated with 6 μM Fura-2/AM (the acetoxymethyl ester of the fluorescent tetracarboxylated chelator Fura-2, Dojin Co., Kumamoto) for about 20 minutes to allow uptake of Fura-2 by the cells. The coverslip was placed in an acrylic rectangular chamber (3.4 x 1.4 x 0.02 cm) so that the cells would be oriented toward the luminal surface. The chamber was a partial modification of the chamber used by Hellumus et al. (34), and the coverslip was fixed to the upper plate of the chamber by negative pressure. The chamber had an entrance and an exit for the solution, and the entrance was connected to a reservoir via a silicone tube. The entire circuit was filled with Krebs buffer bubbled with 5% CO\(_2\)+95% air (glucose 10 mM, KCl 4.8 mM, MgSO\(_4\) 1.2 mM, K\(_2\)HPO\(_4\) 1.2 mM, NaCl 119 mM, NaHCO\(_3\) 24.9 mM, CaCl\(_2\) 2.5 mM) and maintained at 37°C by an automatic temperature controller (Incubator NP2, NIKON). The chamber was placed on the stage of a photometric fluorescence microscope. The cells on the coverslip were irradiated in turn by excitation light beams at 340 nm and 380 nm, and the intensity of the emitted light was measured. After determination in the controls, Krebs solution containing agonists (ACh, BKN, ATP and A23187) in various concentrations (10\(^{-8}\), 10\(^{-7}\), 10\(^{-6}\), 10\(^{-5}\) M) were poured into the chamber through the reservoir at a constant flow rate to stimulate the endothelial cells, and changes in [Ca\(^{++}\); ] were measured. [Ca\(^{++}\); ] was determined from the ratio of fluorescence intensity induced by excitation at one wavelength to the intensity induced by excitation at another wavelength (F340/ F380) using the following formula:

\[
[Ca^{++};] = K_d[(R - R_{min})/(R_{max} - R)](S_1/S_2),
\]

where \(K_d\) is 224 nM, \(R_{max}\) the maximal fluorescence ratio determined after the addition of 10 μM ionomycin and \(R_{min}\) the minimal ratio determined by the subsequent addition of 3 mM Tris ethylene glycol-bis-(β-aminomethyl ether)-N,N’-tetraacetetic acid (EGTA) and 2 μM ionomycin. \(S_1/S_2\) is the ratio of the fluorescence values at 380 nm excitation determined at \(R_{min}\) and \(R_{max}\), respectively. Autofluorescence obtained with 2 mM Mn\(^{++}\) was subtracted from the F340 and F380.

**Bioassay of EDRF.** After intravenous injection of Nembutal (50 mg/kg), 5-6 cm of the thoracic aorta was excised from rabbits (New Zealand white, weighing about 2.5 kg), and 4- to 5-mm rings were prepared. The aortic rings were left standing at 37°C in Krebs buffer bubbled with 5% CO\(_2\)+95% O\(_2\) for about 1 hour, and the endothelial cells inside the rings were removed by brushing with swabs. The aortic rings were stretched to the right and left with a metal wire (diameter, 370 μm) and fixed horizontally. After the tension in the wire had been amplified with an amplifier via a tension sensor (Kyowa Electronic Instruments Co., Ltd., Tokyo), it was recorded.
using a tonograph (Hioki Co., Tokyo). To determine EDRF, the ring was treated with Krebs buffer containing 1 mM norepinephrine (NE) at 37°C at a rate of 3 ml/min after initial loading (1 g) to stabilize the tension, and the aortic ring was continuously constricted maximally. At the same time, Krebs buffer without NE at 37°C was dripped (3 ml/min) onto the ring. Functional removal of endothelial cells from the ring was confirmed by loss of the relaxation response to ACh (10^-5 M). The investigation conforms with the Guide for the care and use of laboratory animals published by the US National Institutes of Health. (NIH publication No 85-23, revised 1985)

Five hundred µl of microcarrier beads were then packed into a polypropylene column (Muromachi Kagaku Kogyo, Ltd., Tokyo) when the cultured cells had become confluent, and the cells were treated with indomethacin (10 µM) to eliminate the effect of endothelium-derived prostacyclin. The column was placed above the aortic ring, application of the buffer without NE was stopped, and 8 ml of Krebs solution containing each of the same agonists as those used in the experiment on Ca^2+ concentration were added at a constant speed to the column containing endothelial cells in order of increasing concentration as follows: ATP (10^-8, 10^-7, 10^-6, 10^-5 M), BKN (10^-8, 10^-7, 10^-6, 10^-5 M), ACh (10^-8, 10^-7, 10^-6, 10^-5 M), and calcium ionophore (A23187, Sigma Chemical Co., St. Louis) (10^-8, 10^-7, 10^-6, 10^-5 M). The effluents were dripped onto the aortic rings (3 ml/min), and their tension was recorded. It took less than 3 seconds for the solution to reach the ring from the uppermost beads in the column. The ratio of the decrease in tension due to the effluent from the column to the tension of the aortic ring constricted with NE was expressed as percentage relaxation, and used as an index of the amount of EDRF produced by the endothelial cells.

**Determination of the content of cGMP in cultured smooth muscle cells.** Smooth muscle cells cultured in the wells of a 6-well plate were incubated for 15 minutes in M199 containing a phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX, 1 mM, Sigma). The medium was removed and the cells were washed in 2 ml of Krebs solution containing 1 mM IBMX. Endothelial cells on beads in the column were stimulated with the same agonists as those in the bioassay experiment, and after 2 minutes, 15 drops (about 1 ml) of the effluent were dripped onto smooth muscle cells within the well. Thirty seconds later, 1 ml of 6% trichloroacetic acid (TCA) was added to the cells, and they were mechanically separated with a cell scraper. After being mixed with a vortex mixer, the cells were cooled in ice. The samples were then centrifuged (at 4°C and 3,000 rpm for 20 minutes), TCA in the supernatant fluid was extracted with water-saturated ether, and they were

### Table 1. Effect of agonists on [Ca^2+]i and EDRF release.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Control</th>
<th>-8</th>
<th>-7</th>
<th>-6</th>
<th>-5</th>
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<tbody>
<tr>
<td>ATP</td>
<td>29.0±0.2</td>
<td>30.1±0.4</td>
<td>50.4±2.7</td>
<td>136.7±22.8</td>
<td>163.0±23.6</td>
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<td>BKN</td>
<td>27.0±1.9</td>
<td>150.2±17.5</td>
<td>102.7±11.2</td>
<td>84.7±6.7</td>
<td>72.9±6.6</td>
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<tr>
<td>ACh</td>
<td>30.1±2.4</td>
<td>28.0±2.1</td>
<td>30.1±2.1</td>
<td>31.1±2.3</td>
<td>30.1±2.2</td>
</tr>
<tr>
<td>A23187</td>
<td>27.0±1.4</td>
<td>36.3±3.1</td>
<td>71.8±4.1</td>
<td>133.4±13.7</td>
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</tbody>
</table>

### % Relaxation

<table>
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<th>-7</th>
<th>-6</th>
<th>-5</th>
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</thead>
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<tr>
<td>ATP</td>
<td>0</td>
<td>7.8±2.5</td>
<td>17.5±8.3</td>
<td>28.1±19.4</td>
<td>35.6±17.8</td>
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<tr>
<td>BKN</td>
<td>0</td>
<td>36.1±2.8</td>
<td>34.3±8.0</td>
<td>30.7±2.9</td>
<td>26.0±4.7</td>
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<tr>
<td>ACh</td>
<td>0</td>
<td>0.5±0.7</td>
<td>1.5±3.1</td>
<td>1.9±5.6</td>
<td>4.9±5.6</td>
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<tr>
<td>A23187</td>
<td>0</td>
<td>5.4±2.6</td>
<td>19.9±2.9</td>
<td>35.3±11.4</td>
<td>44.7±2.8</td>
</tr>
</tbody>
</table>

### cGMP

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Control</th>
<th>-8</th>
<th>-7</th>
<th>-6</th>
<th>-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>17.3±1.1</td>
<td>31.8±7.8</td>
<td>72.2±5.8</td>
<td>134.0±24.0</td>
<td>168.0±28.2</td>
</tr>
<tr>
<td>BKN</td>
<td>19.9±4.1</td>
<td>137.6±32.2</td>
<td>113.7±30.4</td>
<td>97.6±20.0</td>
<td>66.9±17.9</td>
</tr>
<tr>
<td>ACh</td>
<td>17.0±4.7</td>
<td>25.1±6.9</td>
<td>26.3±4.4</td>
<td>26.3±1.6</td>
<td>21.5±9.8</td>
</tr>
<tr>
<td>A23187</td>
<td>19.9±4.1</td>
<td>28.5±4.1</td>
<td>48.1±11.8</td>
<td>113.7±43.7</td>
<td>175.6±35.4</td>
</tr>
</tbody>
</table>

Values are means±SD; n=3 in each experiment. [Ca^2+]i, intracellular Ca^2+ concentration (nM) in endothelial cells; % Relaxation, percent change in the tension of endothelium-free aortic rings maximally contracted with norepinephrine (1 µM); CGMP, cGMP concentration (fmol/10^6 cells) in cultured smooth muscle cells. Concentrations of agonists added to the perfusate of the endothelial cell columns are expressed as logarithm of cumulative molar concentrations. Data were obtained 2 minutes after stimulation of endothelial cells with agonists. There were no significant changes in tension or cGMP levels when these agonists (10^-8-10^-5 M) were directly added to aortic rings constricted by norepinephrine or to cultured smooth muscle cells. No [Ca^2+]i was obtained at 10^-5 M A23187 because [Ca^2+]i became unstable at that concentration. This was believed to be due to leakage of Fura-2 from the cells by A23187, although the exact reason is unclear.
stored at -70°C after lyophilization. cGMP concentration was determined by radioimmunoassay using a [125I] cGMP kit (Amersham International Ltd., U.K.). It was measured according to the acetylation protocol, and the period of incubation after addition of [125I] cGMP was 20 hours. cGMP content expressed as fmol/10^6 smooth muscle cells was used as an index of the amount of EDRF produced by the endothelial cells.

To assess the role of extracellular Ca^++ in EDRF release, endothelial cells were stimulated with ATP (10^{-7} M), BKN (10^{-8} M), or A23187 (10^{-6} M) in Ca^++ free-0.2 mM EGTA Krebs solution, and changes in [Ca^++]i, relaxation response of

![Diagram of calcium concentration and relaxation response](image)

Fig. 1. Left panel: Typical tracings of original recordings of changes in intracellular Ca^++ concentration ([Ca^++]i) when endothelial cells pre-treated with indomethacin (10 ^jM) were stimulated with ATP, BKN, ACh and A23187. All concentrations indicated are expressed as logarithms of cumulative molar. Bars represent the addition of individual doses of agonists. ATP and A23187 increased [Ca^++]i dose-dependently. Initial stimulation with BKN increased [Ca^++]i to about 5 times the resting level, however after that it gradually decreased even when the dose of BKN was increased. ACh caused no changes in [Ca^++]i. Right panel: Typical tracings of original recordings of changes in the tension of endothelium-free aortic rings maximally contracted with norepinephrine (NE, 1 ^{iM}) when the effluent from endothelial cell (EC) columns exposed to agonists was dripped onto aortic rings. Endothelial cells were pretreated with indomethacin (10 ^jM) to eliminate the effect of prostacyclin. The effluent from the cells stimulated with ATP and A23187 induced a dose-dependent relaxation response in aortic rings. When stimulated with BKN, there was marked relaxation of the aortic rings in response to the initial stimulation (10^{-6} M), but at higher concentrations no additional changes were observed. No relaxation responses were observed in aortic rings when stimulated with ACh.
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aortic rings and cGMP levels in cultured smooth muscle cells were examined.

In some experiments, endothelial cells were treated with a specific inhibitor of NO release, Nω-methyl L-arginine (L-NMA, 200 μM) (29) and stimulated with ATP (10^-5 M), BKN (10^-5 M), or A23187 (10^-6 M). L-arginine (1 mM) was then added to the endothelial cells with L-NMA and each agonist. Relaxation response of aortic rings and cGMP contents in cultured smooth muscle cells were examined.

RESULTS

Changes in [Ca^{++}] and in the amount of EDRF released when cultured endothelial cells were stimulated with ATP, BKN, ACh, A23187 are summarized in Table I. Actual [Ca^{++}], after addition of the agonists and actual changes in tension of the aortic rings are shown in Fig. 1.

When endothelial cell were stimulated with ATP,
Fig. 3. Effects of removal of extracellular Ca$^{++}$ on agonist-induced changes in $[Ca^{++}]_{i}$ and EDRF release. EGTA, Ca$^{++}$-free Krebs buffer containing 0.2 mM EGTA; Ca$^{++}$ or buffer, Krebs buffer containing 2.5 mM CaCl$_{2}$; EC, endothelial cells on beads packed in a column; W, wash out. In the absence of extracellular Ca$^{++}$, the agonist-induced continuous increase in $[Ca^{++}]_{i}$ (left panel) and relaxation response in the aortic rings (right panel) observed in the presence of extracellular Ca$^{++}$ was markedly inhibited. EGTA had no effect on the tension of SMC when added directly to the NE-contracted aortic rings. This seemed to be because NE solutions contained enough Ca$^{++}$ to keep the intracellular Ca$^{++}$ levels of SMC almost constant. When SMC were dripped with an effluent containing Ca$^{++}$ and A23187, however, the possibility that A23187 increased $[Ca^{++}]_{i}$ in SMC and attenuated the effect of EDRF cannot be ruled out.
Concentrations of CGMP are expressed as fmol/10^6 cells and values are means±SD; n=3 in each experiment. When BKN was added to endothelial cells at an initial concentration of 10^-8 M, [Ca^{++}]i increased to about 5 times the resting level. However, there was no further increase in [Ca^{++}]i response to additional increases in BKN concentration. Instead, the level gradually decreased. Both percentage relaxation and cGMP levels increased after the initial stimulation (10^-8 M), from 0 to 36.1% and from 19.9 to 137.6 fmol/10^6 cells, respectively. No additional changes were observed at higher concentrations. This phenomenon may be due to desensitization to BKN stimulation (18), although no definite interpretation can be made since studies have not been performed at lower concentrations of BKN (<10^-8 M). When stimulated with ACh, there were no significant changes in [Ca^{++}]i, percentage relaxation or cGMP levels, regardless of the concentration. A calcium ionophore, A23187, dose-dependently increased [Ca^{++}]i, percentage relaxation and cGMP levels.

The relationship between changes in [Ca^{++}]i, and the amount of EDRF released was assessed on the basis of the data described in Table I. As shown in Fig. 2, there was a statistically significant correlation between [Ca^{++}]i and percentage relaxation, and between [Ca^{++}]i and cGMP level, suggesting that the amount of EDRF released is linked to [Ca^{++}]i.

To determine the role of extracellular Ca^{++} in EDRF release, endothelial cells were stimulated with ATP (10^-5 M), BKN (10^-8 M) or A23187 (10^-6 M) in Ca^{++}-free Krebs solution containing 0.2 mM EGTA, and changes in [Ca^{++}]i, and the amount of EDRF released were monitored. As shown in Fig. 3, in the absence of extracellular Ca^{++}, the agonist-induced continuous elevation in [Ca^{++}]i observed in the presence of extracellular Ca^{++} was diminished, although a small peak rise in [Ca^{++}]i was seen. The relaxation response in aortic rings disappeared in the absence of extracellular Ca^{++}. The increase in cGMP induced by the agonists was also blocked by removal of extracellular Ca^{++}, as shown in Table II. These findings suggest that endothelial cells require extracellular Ca^{++} to release EDRF in response to agonists.

To determine whether the EDRF released from endothelial cells was NO, endothelial cells were treated with L-NMA, L-arginine or both. The effects of L-NMA and L-arginine on agonists-induced EDRF release are shown in Fig. 4. L-NMA inhibited agonist-induced EDRF release, and L-arginine partially reversed L-NMA induced inhibition, confirming that EDRF consisted of nitric oxide (NO) or NO-related substances.

### Table II. Effect of Removal of Extracellular Ca^{++} on Agonist-Induced EDRF Release

<table>
<thead>
<tr>
<th>Extracellular Ca^{++}</th>
<th>ATP (10^-5 M)</th>
<th>BKN (10^-8 M)</th>
<th>A23187 (10^-6 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>168.0±28.2</td>
<td>137.6±32.2</td>
<td>113.7±43.7</td>
</tr>
<tr>
<td></td>
<td>30.4±5.7</td>
<td>20.4±3.6</td>
<td>32.2±10.2</td>
</tr>
</tbody>
</table>

Concentrations of cGMP are expressed as fmol/10^6 cells and values are means±SD; n=3 in each experiment. After cultured smooth muscle cells in the wells of a 6-well plate were pretreated with 1 mM isobutylmethylxanthine (IBMX), they were exposed for 30 seconds to the effluents from endothelial cell columns stimulated with agonists in the presence of extracellular Ca^{++}. The increase in cGMP observed in the presence of extracellular Ca^{++} almost disappeared in its absence. This suggests that extracellular Ca^{++} is essential for agonist-induced EDRF release. The control level of cGMP in cultured smooth muscle cells not stimulated with any agonists was 22.1±1.8 fmol/10^6 cells (n=6). Basal [Ca^{++}]i decreased from 27.7±1.2 to 15.0±0.6 nM when EC were incubated with Ca^{++}-free Krebs buffer containing EGTA.

**DISCUSSION**

A substance which relaxes rabbit aortic rings was released in response to stimulation of cultured endothelial cells by the agonists ATP, BKN and A23187. Endothelial cells are known to produce and release prostacyclin (23), endothelium-derived relaxing factor (EDRF) (11) and endothelium-derived hyperpolarization factor (EDHF) (15). Since the endothelial cells had been treated with the cyclooxygenase inhibitor indomethacin in advance, prostacyclin can be excluded as the substance inducing vascular relaxation. In the case of EDHF, on the other hand, its nature still remains unknown, and since the membrane potential of aortic ring smooth muscle was not measured in the present experiment, it is impossible to determine whether or not it is involved in the relaxation response of the aortic rings. However, unlike EDRF, EDHF is not believed to increase intracellular CGMP (16, 38), even when it acts on smooth muscle cells. Thus, EDHF can be excluded as a substance which induces vascular relaxation and increases cGMP in cultured smooth muscle cells. Furthermore, although ATP and BKN may cause hyperpolarization of endothelial cells (19), it is impossible for changes in the membrane potential of endothelial cells to directly affect the membrane potential of the smooth muscle to induce relaxation, because cultured endothelial cells on beads in the column are spatially apart from the smooth muscle of the aortic rings. Based on the above findings, the substance that relaxed the aortic rings and increased cGMP in cultured smooth muscle cells in the present study is believed to be EDRF. The release of EDRF by stimulations with ATP, BKN and A23187 was almost completely blocked by a specific NO release inhibitor, L-NMA. The addition of L-arginine...
reversed the L-NMA-induced inhibition. Thus the EDRF released from cultured fetal bovine aortic endothelial cells was considered to be NO or NO-related substances.

The results of the present study suggest that changes in extracellular or intracellular Ca^{2+} are closely involved in EDRF release from endothelial cells by agonists. Stimulation of cultured endothelial cells with ACh, which caused no changes in [Ca^{2+}], did not increase the amount of EDRF released. This observation is consistent with the findings reported by Cocks et al. (9). It is possible that the muscarinic receptor or its coupling to subsequent biochemical events is lost in culture. An alternative explanation proposed by Stephenson and Summers (35) is that as muscarinic receptors cannot be found on the endothelium of a wide range of blood vessels by autoradiographic techniques, endothelium-dependent relaxation might result from an indirect mechanism through receptors located on the smooth muscle. On the other hand, ATP, BKN and A23187, which increased [Ca^{2+}], enhanced the release of EDRF by endothelial cells. An influx of extracellular Ca^{2+} is known to be involved in the continuous increase in [Ca^{2+}], induced by these agonists (20). As mentioned above, when extracellular Ca^{2+} was excluded, the agonist-induced continuous increase in [Ca^{2+}] was markedly suppressed, and at the same time the EDRF release response to agonists almost disappeared. These results are in accordance

Fig. 4. Effects of L-NMA and L-arginine on agonist-induced EDRF release. Values are means±SD; n=3 in each experiment. L-NMA or L-arginine had no effect on the tension or cGMP levels when they were directly added to aortic rings constricted by NE or to cultured smooth muscle cells.
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with an *in vivo* phenomenon in rabbits and rats (13, 17, 38) in which EDRF release from the endothelium is dependent on extracellular Ca++. Schmidt et al. (33), however, observed that EDRF production, estimated on the basis of cGMP levels in endothelial cells, occurred when endothelial cells were stimulated with agonists even in the absence of extracellular Ca++. On the basis of these findings, the investigators suggested the following concept: Ca++ elevation caused by its release from sites of intracellular Ca++ storage, such as the endoplasmic reticulum, is necessary for the initiation of EDRF production by endothelial cells in response to agonists, but an influx of extracellular Ca++ is not always required. Therefore, the role of Ca++ might need to be considered in differentiating the initiation of production from the continuous release of EDRF.

It has been shown that the presence of extracellular Ca++ is essential for the continuous release of EDRF by agonists (37), but there have been no reports of a quantitative relationship between changes in [Ca++] and the production and release of EDRF. In the present study, stimulation of endothelial cells with ATP and A23187 increased [Ca++] dose-dependently at the same time that it increased the amount of EDRF released. Although no precise temporal relationship was found because the Ca++ concentration was not determined simultaneously with the concentration of EDRF in the same cells, the course of changes in one of the parameters coincided with changes in the other. Changes in both the Ca++ concentration and the amount of EDRF released started immediately after endothelial cells were stimulated with ATP and A23187 and when the concentrations changed. EDRF was not released in the absence of an increase in the [Ca++] . Conversely, [Ca++] was increased by stimulation with ATP and A23187, even when endothelial cells were treated with L-NMA to suppress EDRF production (data not shown). These findings suggest that the increase in Ca++ concentration is not due to the EDRF produced in the cells.

An enzyme converting L-arginine to an activator of guanylyl cyclase, i.e., NO synthase, has recently been purified from rat, porcine and bovine cerebellum and characterized (4, 21, 26). There are at least two distinct types of NO synthase: the constitutive type, whose activity is insensitive to changes in intracellular Ca++ and which requires tetrahydrobiopterin and NADPH as cofactors. It was also shown that there is NO synthase in endothelial cells (5, 6) and that the activity of the enzyme depends on [Ca++] within the range of 1 nM to 1 μM (21). Taking this report into consideration along with our own current findings, it is very likely that the constitutive type of NO synthase participates in the mechanism by which EDRF release from cultured fetal bovine aortic endothelial cells is directly linked to intracellular Ca++ concentration. However, many questions remain as to the role of Ca++ in the production and release of EDRF. For example, it has not been elucidated how Ca++ enhances NO synthase activity, whether mechanisms for the stimulated release of EDRF from endothelial cells are always dependent on Ca++, what the role of calcium-dependent activation in changes of EDRF activity is, whether the effect of Ca++ is dependent solely on calmodulin-dependent stimulation of basal NO synthase activity or whether non-calmodulin dependent mechanisms are also involved. Further study will be necessary to answer these questions.

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