Contribution of Sustained Ca\(^{2+}\) Elevation for Nitric Oxide Production in Endothelial Cells and Subsequent Modulation of Ca\(^{2+}\) Transient in Vascular Smooth Muscle Cells in Coculture\(^*\)

(Yuepeng Wang, Wee Soon Shin, Hiroyuki Kawaguchi, Masao Inukai, Masayoshi Kato, Aiji Sakamoto, Yoshio Uehara, Miwa Miyamoto, Norio Shimamoto, Risa Korenaga, Joji Ando, and Teruhiko Toyo-oka)

From the Second Department of Internal Medicine, Health Service Center, the Department of Biomechanics, University of Tokyo, Tokyo 113, and the Molecular Chemistry Laboratory, Takeda Chemical Industries, Osaka 532, Japan.

To elucidate the intracellular Ca\(^{2+}\) transient (Ca\(^{2+}\)i) transient responsible for nitric oxide (NO) production in endothelial cells (ECs) and the subsequent Ca\(^{2+}\)i reduction in vascular smooth muscle cells (VSMCs), we administrated four agonists with different Ca\(^{2+}\)i-mobilizing mechanisms for both cells in iso- or coculture. We monitored the Ca\(^{2+}\)i of both cells by two-dimensional fura-2 imaging, simultaneously measuring NO production as peak Ca\(^{2+}\)i reduction in cocultured VSMCs and the elevation in NO production over the level of basal release in ECs completely matched and was ranked as thapsigargin > ionomycin > ATP > bradykinin. Treatment by N\(^{0}\)-monomethyl-L-arginine monoacetate but not indomethacin or glybenclamide restored the Ca\(^{2+}\)i response in cocultured VSMCs to the isoculture level. In ECs, when the Ca\(^{2+}\)i influx was blocked by Ni\(^{2+}\) or by chelating extracellular Ca\(^{2+}\), all four agonists markedly decreased NO production, the half decay time of the Ca\(^{2+}\)i, degenerating phase, and the area under the Ca\(^{2+}\)i curve. The amount of produced NO hyperbolically correlated to the half decay time and the area under the Ca\(^{2+}\)i curve but not to the Ca\(^{2+}\)i, peak level. Thus, the sustained elevation of Ca\(^{2+}\)i in ECs, mainly a result of Ca\(^{2+}\)i influx, determines the active NO production and subsequent Ca\(^{2+}\)i reduction in adjacent VSMCs. Furthermore, L-arginine but not D-arginine or L-lysine at high dose (5 mM) without agonist enhanced the NO production, weakly reduced the Ca\(^{2+}\)i in ECs, and markedly decreased the Ca\(^{2+}\)i in VSMCs, demonstrating the autocrine and paracrine effects of NO (Shin, W. S., Sasaki, T., Kato, M., Hara, K., Seko, A., Yang, W. D., Shimamoto, N., Sugimoto, T., and Toyo-oka, T. (1992) J. Biol. Chem. 267, 20377-20382).

Endothelial cells (ECs) modulate the contractility of underlying vascular smooth muscle cells (VSMCs), secreting several vasoconstrictors and vasorelaxants (1, 2). The principle endothelium-derived relaxing factors have been identified as nitric oxide (NO) (3) and prostacyclin. Both are regulated by intracellular calcium ions (Ca\(^{2+}\i\)) (4). In the absence of extracellular Ca\(^{2+}\i\) (Ca\(^{2+}\i\)), NO production is greatly reduced (5, 6). Recently, we have presented evidence that NO affects the handling of Ca\(^{2+}\)i by an autocrine action in NO-producing ECs and by a paracrine action in adjacent VSMCs (7). However, little quantitative information is available on the relationship between the Ca\(^{2+}\)i in ECs and NO production. There is also a lack of information regarding the amount of NO produced in ECs and its action to reduce the Ca\(^{2+}\)i levels in neighboring VSMCs.

The Ca\(^{2+}\)i in VSMCs is crucial, because it is a primary factor in the regulation of muscle contractility (8). Most Ca\(^{2+}\)i transients in ECs induced by agents that cause the release of NO consist of a peak followed by a degeneration phase (5). The peak originates from the release of Ca\(^{2+}\i\) from endoplasmic reticulum. The influx of Ca\(^{2+}\)i from the extracellular medium accounts for the maintenance of the subsequent portion of degeneration phase. To determine which component of the Ca\(^{2+}\i\) transients of ECs is most significant in indicating the production of NO and to monitor the biological action of NO on VSMCs, we simultaneously measured the stable NO metabolite, nitrite (NO\(_2\)\i\), in the medium and the Ca\(^{2+}\)i of both ECs and VSMCs in coculture by two-dimensional image analysis. We report here a unique communication between the Ca\(^{2+}\)i in ECs and the Ca\(^{2+}\)i in VSMCs mainly mediated by NO.

MATERIALS AND METHODS

Reagents—All reagents used were of analytical grade. Phosphate-buffered medium was utilized as the extracellular medium and was composed as follows: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na\(_2\)HPO\(_4\), 1.5 mM KH\(_2\)PO\(_4\), 1.0 mM CaCl\(_2\), and 0.5 mM MgCl\(_2\), pH 7.4. Ca\(^{2+}\)-free medium consisted of phosphate-buffered medium in which CaCl\(_2\) was replaced by EGTA (1.0 mM). When Ni\(^{2+}\) was employed in our protocol, the medium was switched to HEPES (Doshin, Tokyo) buffer (145 mM NaCl, 5.0 mM KCl, 1.0 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 10 mM d-glucose, and 10 mM HEPES, pH 7.4) to prevent a precipitation in phosphate-buffered medium caused by the addition of Ni\(^{2+}\). Thapsigargin (TG, Sigma) and ionomycin (IM, Sigma) were dissolved in Me\(_2\)SO and then diluted with buffer. The final concentration of Me\(_2\)SO was less than 0.1% (v/v) and therefore had no direct action on the handling of Ca\(^{2+}\)i in either ECs or VSMCs.

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¶ To whom correspondence should be addressed: Second Dept. of Internal Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, J. Japan. Tel.: 81-3-3815-5411, ext. 3074; Fax: 81-3-3813-2009.

1 The abbreviations used are: EC, endothelial cell; VSMC, vascular smooth muscle cell; NO, nitric oxide; TG, thapsigargin; IM, ionomycin; L-NMMA, N\(^{\text{0}}\)-monomethyl-L-arginine monoacetate; BK, bradykinin; HDT, half decay time; AUC, area under the Ca\(^{2+}\)i curve; HPLC, high pressure liquid chromatography; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; CaM, calmodulin.
Ca\(^{2+}\) Signal and Nitric Oxide Production in Endothelial Cells

VSMCs. N\(^{6}\)-monomethyl-L-arginine monooacetate (L-NMMA) was purchased from Calbiochem (La Jolla, CA). Bradykinin (BK), ATP, glybenclamide, and indomethacin were from Sigma. L-Arginine monohydrochloride, D-arginine monohydrochloride, and L-lysine monohydrochloride were from Wako (Tokyo, Japan). Each agent was administered by replacing half of the volume of extracellular medium with a twice concentrated solution of the reagents in incubation medium.

Isoculture or Coculture of ECs and VSMCs—ECs were enzymatically isolated from bovine aorta and then cultured and identified as described previously (7). They were seeded in two kinds of dishes, one made of a fluorescence-free glass for assaying both Ca\(^{2+}\), and NO\(_{2}^-\) (1 ml in volume) and another made of plastic for the periodic sampling and measurement of NO\(_{2}^-\) (10 ml in volume). A cell line of VSMCs, A7r5, from American Type Culture Collection (Rockville, MD) was cultured using the same method as ECs.

To assess the biological effect of NO on the Ca\(^{2+}\), in VSMCs, we cultured ECs and VSMCs together (7). Three days after the seeding of VSMCs, at a density of 1.5 × 10\(^3\)/cm\(^2\), the culture dishes were semiconfluent. ECs were then added to the culture at the same initial density of VSMCs, 1.5 × 10\(^3\)/cm\(^2\). Under these conditions, both VSMCs and ECs grew symbiotically.

Measurement of Ca\(^{2+}\) by Two-dimensional Image Analysis—Ca\(^{2+}\) within individual cells was analyzed as described previously (9, 10). Significant leaking of fura-2 did not occur during the measurement, evidenced by the increment of autofluorescence in the incubation medium. All procedures including the addition of drugs to the medium were performed under dark conditions, and illumination time was minimized to prevent the photobleaching of fura-2. Because the fura-2 method has several intrinsic problems in the estimation of absolute Ca\(^{2+}\) (11), the amplitude of Ca\(^{2+}\) elevation in response to each stimulant was calculated using the percentage of increase of F340/F380 of the cell.

In a pilot study for dose determination, we searched for suitable agonists and their appropriate concentrations that make it possible to analyze Ca\(^{2+}\), transients using different receptor/effector coupling systems. Iso- or cocultured ECs or VSMCs were stimulated by ATP (10 \(\mu\)M) or BK (100 nM) for 3 min. In the cases of TG (1 \(\mu\)M) and IM (50 nM), the stimulation time was extended to 40 min because the Ca\(^{2+}\) response to these agonists was much more gradual than ATP or BK. To quantify the Ca\(^{2+}\) responses in ECs, we measured three parameters: the peak level, the half decay time (HDT), and the area under the Ca\(^{2+}\) curve (AUC) by computer-assisted planimetry as shown in Fig. 1. To examine the reproducibility of Ca\(^{2+}\) responses to the same agonist, a second stimulation was performed after a 30-min equilibration period following the washout of the first application of the agonist.

Data Analysis—All data were expressed by the mean ± S.E. The difference between responses was analyzed either by paired t test or by analysis of variance. All differences were considered statistically significant if p < 0.05.

RESULTS

NO Production in ECs in Isoculture

Time Course of NO Production—The time course of NO production was determined by analyzing aliquots obtained from ECs cultures in plastic dishes containing 10 ml of medium. Under control conditions with no addition of agonist, NO assayed as NO\(_{2}^-\) was found to be present 10.5 ± 0.6, 11.1 ± 1.1, and 11.4 ± 1.1 ng/ml/10\(^6\) cells just before or 3 or 40 min after starting the incubation, respectively. These results were likely caused by the shear stress during the exchange of the medium rather than a gradual release that occurred throughout the

FIG. 1 Measurement of peak, HDT, and AUC.
incubation, because NO was not accumulated significantly after 40 min (Fig. 2, Ctl). After the administration of ATP, the level of NO (ng/ml/10^6 cells) increased from 10.9 ± 0.4 to 13.4 ± 0.8 ng/ml/10^6 cells (p < 0.01) at 3 min; adding BK to the medium resulted in a rise from 10.7 ± 0.6 to 12.4 ± 0.8 ng/ml/10^6 cells at 3 min (p < 0.05, Fig. 2A). In the experiments involving ATP and BK, measurements were taken at the end of a 3-min incubation after which the production of NO became stable. After an incubation for 40 min, the level of NO steadily increased from 10.5 ± 0.5 to 23.8 ± 1.5 (p < 0.01) in response to TG and from 10.9 ± 0.5 to 14.0 ± 0.4 ng/ml/10^6 cells (p < 0.01) in response to IM (Fig. 2B).

Dependence of NO Production on Ca^2+ Influx and Effect of Substrate—Another experiment showed that NO production is dependent on the entry of Ca^2+ into ECs. Agonist-induced NO production was enhanced relative to basal release by all four agonists in the presence of 1 mM Ca^2+. However, when Ca^2+ influx was chelated by 1 mM EGTA without adding Ca^2+ to the phosphate-buffered medium, ATP, BK, and TG caused production of lower amounts of NO, close to or less than basal release. Furthermore, in media containing 1 mM Ca^2+, the nonspecific divalent cation channel blocker, Ni^2+ (1 mM), the agonist-induced NO production was also reduced to basal or near basal level release (Fig. 3A).

To verify the agonist-induced NO production, we used the NO synthase inhibitor, L-NMMA. Pretreatment by L-NMMA (500 μM, 30 min) inhibited NO production over the basal level induced by all four agonists. However, the addition of L-arginine (5 mM) negated this inhibition and increased the production of NO after administration of all four agonists, ranging from 2-fold in the case of BK to 8-fold in the case of TG (Fig. 3B). These results indicate that NO was synthesized from L-arginine.

### Ca^2+ Transients

**ECs in Iso- and Coculture**—In the presence of Ca^2+, all four agonists raised the Ca^2+ in cocultures of ECs, demonstrating different peak levels and degeneration phases (Fig. 4). Among the agonists, the Ca^2+ levels was highest by ATP and BK, peaking at 20 s. Compared with ATP (10 μM), BK (100 nM) prominently raised the Ca^2+ level, which then decreased at a faster rate (Fig. 4A). In contrast, the Ca^2+ change was more gradual when caused by either IM or TG. Both of these agonists raised the Ca^2+ level gradually after stimulation by TG or IM, the recording time was extended to 2400 s (B). Each point denotes the mean ± S.E. (n = 130–147).

**Fig. 4. Ca^2+ dynamics in isocultured ECs induced by four kinds of agonist with different Ca^2+ mobilizing actions.** At the arrow, ATP (10 μM, ●), BK (100 nM, ○), TG (1 μM, □), or IM (50 nM, ▲) was added in the presence of 1 mM Ca^2+, and the Ca^2+ was monitored (A; Ref. 7). To show the later phase after stimulation by TG or IM, the recording time was extended to 2400 s (B). Each point denotes the mean ± S.E. (n = 130–147).
Table II). The Ca\(^{2+}\) (100 nM) raised the Ca\(^{2+}\) afterstimulation. The Ca\(^{2+}\) agonist.

Fig. 6). In addition, the ATP-induced Ca\(^{2+}\) showed the later phase afterstimulation by TG (1

### Table I

| Ca\(^{2+}\) in Isoculture | Ca\(^{2+}\) in Coculture | ECs in Isoculture | ECs in Coculture |
|--------------------------|--------------------------|------------------|------------------|
| **Peak HDT AUC** | **Peak HDT AUC** | **Peak HDT AUC** | **Peak HDT AUC** |
| Ca\(^{2+}\) (+) | Ca\(^{2+}\) (-) | Ca\(^{2+}\) (+) and Ni\(^{2+}\) (+) | Ca\(^{2+}\) (+) and Ni\(^{2+}\) (+) |
| ATP | 81 ± 4 | 67 ± 10 | 9655 ± 754 | 56 ± 8 |
| BK | 112 ± 6 | 103 ± 10 | 12712 ± 1007 | 98 ± 6 |
| TG | 58 ± 3 | 600 ± 20 | 52102 ± 360^a | 46 ± 4^b |
| IM | 70 ± 4 | 187 ± 14 | 46812 ± 3774^o | 71 ± 7 |

a Denotes statistically significant from Ca\(^{2+}\) (+) value at p < 0.05.

b Denotes statistically significant from ATP value at p < 0.05.

Identification of the Ca\(^{2+}\) Modulating Factor as NO in Coculture

The following four findings strongly support our scheme that the Ca\(^{2+}\) reduction in cocultured VSMCs is principally regulated by NO derived from ECs: (i) A pretreatment by the NOS inhibitor, L-NMMA (500 μM), for 30 min attenuated the Ca\(^{2+}\) decrease in VSMCs caused by ATP and BK (Table II). The same treatment abolished the Ca\(^{2+}\) reduction in VSMCs after application of TG and IM, restoring the response to control levels (Fig. 9 and Table II); (ii) A pretreatment by the cyclooxygenase inhibitor, indomethacin (50 μM), for 15 min had no effect on the EC-dependent Ca\(^{2+}\) decrease in VSMCs caused by the four agonists (Table II). These results suggest that prostaglandins were not involved in the reduction of Ca\(^{2+}\) in VSMCs cocultured with ECs; (iii) In the case of ATP, the 30-min pretreatment by a specific inhibitor to ATP-sensitive K\(^+\) channels, glibenclamide (10 μM), showed no effect on the ATP-induced Ca\(^{2+}\) reduction in cocultured VSMCs (data not shown). (iv) Surprisingly, a high concentration of L-arginine (5 mM) without agonist induced a moderate Ca\(^{2+}\) decrease to below basal level in ECs and a profound reduction of Ca\(^{2+}\) in VSMCs (Fig. 11, C and D) accompanied by a large amount of NO production (Fig. 11E). Interestingly, the L-arginine-induced NO production was not dependent on Ca\(^{2+}\) because a similar NO level was produced in both the presence and the absence of Ca\(^{2+}\) in cocultured ECs (Fig. 11E). These results are evidence of an autocrine effect of NO on ECs that reduces their Ca\(^{2+}\) and a paracrine effect on VSMCs in coculture (7), whereas D-arginine (5 mM) and L-lysine (5 mM) did not induce any Ca\(^{2+}\) reduction in cocultured VSMCs (data not shown).

(100 nm) raised the Ca\(^{2+}\), only very weakly (8 ± 3% at 5 s and 2 ± 1% at 3 min; Fig. 5A), although the response was much stronger when a high dose (10–100 μM, data not shown) was used. TG (1 μM) induced a unique Ca\(^{2+}\) response that showed a shoulder at 1 min (60 ± 7%), peaked at 10 min (107 ± 5%), and then declined gradually up to 40 min (16 ± 2%; Fig. 5B) after stimulation. The Ca\(^{2+}\) rise induced by IM (50 nm) became evident at 1 min, peaked at 4 min, and then slowly decreased (43 ± 2% at 40 min; Fig. 5B).

When VSMCs were cocultured with ECs, these Ca\(^{2+}\) changes in VSMCs were strikingly modified (Figs. 6–10 and Table II). The Ca\(^{2+}\) in VSMCs after ATP stimulation did not increase but decreased to below basal level (−21 ± 3% at 3 min; Fig. 6). In addition, the ATP-induced Ca\(^{2+}\), transient in cocul-
Each value denotes the mean ± S.E. (n = 28–78 in isoculture; n = 45–85 in coculture, respectively). To quantify the effect of culture conditions on the Ca\(^{2+}\) dynamics, the difference of each parameter in isoculture was subtracted from that in coculture (Difference of parameters). The peak of Ca\(^{2+}\) (Peak) was expressed as the percentage of change over the basal level and the AUC in %s.

### Table II

| Isoculture | Coculture | Difference of parameters | Coculture + l-NMMA treatment | Coculture + indomethacin treatment |
|------------|-----------|--------------------------|-------------------------------|-----------------------------------|
| Peak AUC   | Peak AUC  | Peak AUC                 | Peak AUC                      | Peak AUC                          |
| ATP 86 ± 6 | 12355 ± 761 | 21 ± 3                  | 107 ± 4                       | 13985 ± 547                       | 25 ± 9a                  | 3272 ± 446a                | −17 ± 4                  | −1553 ± 432                |
| BK 9 ± 2b  | 637 ± 2535 | −5 ± 1b                 | 14 ± 2b                       | 1189 ± 223b                       | 6 ± 2b                   | 1137 ± 543b                | −5 ± 3b                  | −528 ± 156b                |
| TG 107 ± 5b | 128428 ± 8850 | −25 ± 3b                   | 132 ± 3b                       | 175474 ± 9490b                    | 92 ± 12b                 | 117344 ± 6312b             | −22 ± 8                  | −39124 ± 7754               |
| IM 90 ± 4  | 141936 ± 6432 | 21 ± 3b                   | 70 ± 3b                       | 108712 ± 4117b                    | 61 ± 8b                  | 123727 ± 4433b             | 19 ± 5b                  | 34534 ± 3314b               |

*Denotes statistically significant from Coculture value at p < 0.05.

**Denotes statistically significant from ATP value at p < 0.05.

FIG. 6. **Two-dimensional images of the Ca\(^{2+}\) response of ECs and VSMCs in coculture (×200) induced by ATP with or without Ca\(^{2+}\) and the Ca\(^{2+}\) dynamics in both cells.** The experiments show the positions of ECs and VSMCs (A), fura-2-loaded cultured cells in the resting state at an excitation wavelength of 380 nm (B), and peak F340/F380 ratio image in the presence (C) or the absence (D) of Ca\(^{2+}\), after stimulation by ATP (10 μM). At the arrow, ATP was added. The second dose of ATP was applied 30 min after the first dose. The Ca\(^{2+}\) transient of ECs (●) and VSMCs (○) with (E) or without (F) Ca\(^{2+}\). Each point denotes the mean ± S.E. (n = 6–8). The bar represents 100 μm.

**Correlations between NO Production and Ca\(^{2+}\) Transients**

The ATP- or BK-induced NO\(_2\) rise stabilized 3 min after stimulation (Fig. 2A), and the Ca\(^{2+}\) response began to decline at the end of 3 min (Fig. 4A). TG- or IM-induced NO production continued to increase up to 40 min after stimulation (Fig. 2B), and simultaneous recording of the Ca\(^{2+}\) showed a decrease up to the 40-min time point (Fig. 4B). Accordingly, we have chosen an incubation time of 3 min for ATP- or BK-induced NO\(_2\) production and of 40 min for TG- or IM-induced NO\(_2\) production to correlate with the Ca\(^{2+}\) pattern.

As summarized in Fig. 12A, the NO production increased sharply as the HDT rose to 200 s. After that time, further increments of NO\(_2\) production became reduced. The AUC was plotted in Fig. 12B. The amount of NO\(_2\) sharply increased until the AUC reached 20,000% and then gradually increased. Accordingly, the amount of NO\(_2\) hyperbolically correlated to both the HDT of Ca\(^{2+}\) (r = 0.90, p < 0.001) and the AUC (r = 0.86, p < 0.001) but did not correlate to the peak Ca\(^{2+}\) in ECs (r = 0.17, p > 0.05; Fig. 12C). These results suggest that the NO production rate was saturated by a long sustained Ca\(^{2+}\) augmentation.

**DISCUSSION**

This is the first report to describe that (i) NO is released from ECs at the basal level even without the presence of agonist, (ii) the maintenance of Ca\(^{2+}\) level, which is supported by Ca\(^{2+}\) influx, determines the rate of NO production over the basal release in ECs, (iii) NO production is not related to the peak Ca\(^{2+}\) but dependent on the sustained elevation of Ca\(^{2+}\), as represented by the HDT or AUC of Ca\(^{2+}\) in ECs, (iv) the production of NO reduces the Ca\(^{2+}\) in cocultured VSMCs in a paracrine manner, dependent on the level of NO production, and (v) l-arginine alone enhances NO production, which has both autocrine and paracrine actions on ECs and VSMCs in coculture, respectively.

**Methodologies of NO Assay—** The best method to quantify the NO production in ECs would be a direct and real-time measurement of NO, using an electrode specifically sensitive to NO. We tested several electrodes that have been reported to be effective (13–15) but failed to exactly measure the basal NO production of NO, using an electrode specifically sensitive to NO. We tested several electrodes that have been reported to be effective (13–15) but failed to exactly measure the basal NO release and the additional production of NO after agonist application, because of the insufficient sensitivity. Very recently, Malinski’s group has used BK for the simultaneous measurement of both Ca\(^{2+}\) and L-NMMA treatment. In this measurement, the Ca\(^{2+}\) transient was actually dependent on the Ca\(^{2+}\) level, which is supported by Ca\(^{2+}\) influx, determines the rate of NO production over the basal release in ECs, (iii) NO production is not related to the peak Ca\(^{2+}\) but dependent on the sustained elevation of Ca\(^{2+}\), as represented by the HDT or AUC of Ca\(^{2+}\) in ECs, (iv) the production of NO reduces the Ca\(^{2+}\) in cocultured VSMCs in a paracrine manner, dependent on the level of NO production, and (v) l-arginine alone enhances NO production, which has both autocrine and paracrine actions on ECs and VSMCs in coculture, respectively.

**Previously, two indirect methods have been employed to quantify the NO production in ECs.** One involves the measurement of the isometric tension developed in vessel strips in the presence or absence of ECs and the subsequent comparison of the extent of the vessel relaxation. However, the NO effect cannot be evaluated correctly in this measurement, because the vessel does not contract in the absence of Ca\(^{2+}\). Another method relies on the measurement of intracellular cGMP (17, 18), which is synthesized after the activation of guanylate cyclase. cGMP does not, however, explain all actions of NO (19). Both methods cannot simultaneously measure the Ca\(^{2+}\) dy-
dynamics in ECs and/or VSMCs. No data have been reported concerning the extent of Ca\textsuperscript{2+} elevation in ECs relative to NO production rate or the Ca\textsuperscript{2+} reduction in VSMCs.

Present NO\textsubscript{2} measurement was used to assay NO production mediated by inducible NOS (12), where the amount of released NO was much larger than that from eNOS and accordingly can be easily assayed. The amount of NO\textsubscript{2} in the culture medium of ECs is not measurable by a simple spectrophotometry, because of its insufficient sensitivity. NO\textsubscript{2} determination by HPLC, with enhancing the sensitivity after the oxidation of NO to NO\textsubscript{2} (20), has made it possible to document the trace quantities of NO released from ECs mediated by eNOS for the first time. In this study, ATP and BK induced no fold increase in NO production. However, fold increase in Ca\textsuperscript{2+}, or cGMP induced by ATP, BK, or A23187 as identified by Schmidt et al. (21) did not mean that the rise of NO production must be also in fold. In contrast, there was a marked and continuous rise in NO production by TG and IM in spite of the lower Ca\textsuperscript{2+} rise. These results proved that our method is reliable, although the NO measurements still required multiple sampling to minimize variation between experiments.

In addition to the NO determination, we employed biological action of native NO produced in ECs, because NO reduces the Ca\textsuperscript{2+} in cocultured VSMCs in a paracrine manner (7), dependent on the level of NO production. Using both the NO determination and the bioassay, we succeeded for the first time in developing the quantitative aspect of Ca\textsuperscript{2+} component responsible for NO production but also Ca\textsuperscript{2+} reduction in VSMCs that leads to the muscle relaxation.

Selection and Dose Determination of Agonists—All four agonists used in this study raised the Ca\textsuperscript{2+}, by different mechanisms and caused different peak Ca\textsuperscript{2+} levels and degeneration phases both in ECs and in VSMCs. ATP and BK provoked a Ca\textsuperscript{2+} rise in ECs through P\textsubscript{2y} and B\textsubscript{2} receptors, respectively (22). The present results showing that ATP is more potent than BK are consistent with a previous report in its relationship to cGMP concentration in ECs (21). TG is a specific inhibitor of the Ca\textsuperscript{2+} pump on endoplasmic reticulum and sarcoplasmic reticulum but is not an inhibitor of the Ca\textsuperscript{2+} pump on the plasma membrane or of other ion-mobilizing ATPases (23). TG might increase the Ca\textsuperscript{2+} through a Ca\textsuperscript{2+} influx following the depletion of the internal stores (24, 25). This influx could be mediated by the Ca\textsuperscript{2+} influx factor (26). IM is a Ca\textsuperscript{2+} ionophore and might nonspecifically elevate the Ca\textsuperscript{2+} by the influx.

The doses of these agonists were adjusted to appropriately raise the Ca\textsuperscript{2+} in ECs and produce NO within the measurable range of our system. For those results, we selected the doses determined in a preliminary study according to the following three criteria: (i) The maximum dose was not used to avoid eliciting a saturated response in both ECs and VSMCs and to accurately evaluate L-NMMA or indomethacin. The EC\textsubscript{50} for each agonist varied between ECs and VSMCs. For example, BK induced a larger response in ECs (EC\textsubscript{50} = 30 nM) than in VSMCs (EC\textsubscript{50} = 12 nM). BK at 100 nM caused a significant response in ECs but a weak response in VSMCs. In contrast, the response to IM was stronger in VSMCs (EC\textsubscript{50} = 7 nM) than
in ECs (EC50 = 24 nM). The appropriate range of concentrations of IM was narrow for the Ca2+ measurement. (ii) Agonists should homogeneously excite ECs. All ECs responded to TG and IM, but only 80–85% of the cells responded to ATP at 100 nM (EC50 = 400 nM) or BK at 10 nM. The lower the concentration of ATP or BK, the fewer the number of cells able to respond. The latent time from agonist application to the onset of the Ca2+ rise was more variable at the lower doses, (iii) The agonist concentration chosen should not induce the Ca2+ oscillation that makes the HDT measurement difficult. Low concentrations of ATP causes the oscillation, as reported by Lynch et al. (27). We used 10 μM ATP that produced an exponential degeneration after the peak. BK rarely induced the oscillation, and TG or IM caused no oscillation at all.

**Ca2+ Component in ECs Responsible for NO Production**—The amount of NO production after the administration of each agonist was significantly higher in the presence of Ca2+ than in its absence (Fig. 3A). Many studies have shown that NO production nearly ceased without Ca2+ (5, 6). The present study has indicated that the Ca2+ release only from internal stores, as in the absence of Ca2+-, still has a small but significant effect on the actual production of NO after stimulation of ATP or IM (Fig. 3A), as also revealed by the blunt rise of Ca2+ in cocultured VSMCs (Fig. 5A versus Fig. 6F). The cause of the discrepancy might be due to the lower sensitivity of NO action, including cGMP assay (5, 6).

Furthermore, the quantification of sustained Ca2+ elevation by the HDT or AUC revealed that the NO production rate was hyperbolically correlated to these two parameters but not to the peak Ca2+ level at all (Fig. 12). The simple Ca2+-calmodulin (CaM) scheme would not be applicable for the eNOS activation, because the scheme assumes a homogenous distribution of Ca2+, CaM, and eNOS. In the Ca2+ signaling mediated by inositol 1,4,5-trisphosphate where ATP and BK are concerned, the peak Ca2+ is supplied from the release of Ca2+ from internal stores, independent of the Ca2+ influx (Fig. 3A and Fig. 6F). After TG and IM stimulation, the peak Ca2+ would not be formed by the release of Ca2+ from internal stores but chiefly by the entry of extracellular Ca2+, as confirmed by the delay of peak time between in the presence (Fig. 4) and the absence of Ca2+- or the Ni2+ treatment.2 The Ca2+ release from internal stores accounts for only 1⁄3–1⁄8 of the Ca2+ entry, as shown by the AUC (Table I). These results also suggest that the Ca2+ release from internal stores play a less significant role in elevating the Ca2+ than the Ca2+ influx from extracellular source in the cases of TG and IM stimulation. Compartmentation of either the Ca2+– or NOS enzymes might explain the dissociation between peak Ca2+ and NO production; eNOS is located on the cytoplasmic membrane (28), where the entered Ca2+ may di-

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The activation of eNOS in ECs as well as constitutive NOS in brain is strictly Ca\(^{2+}\)-CaM-dependent. Compared with the binding of inducible NOS to CaM, the binding of eNOS to CaM is loose and reversible (29). The Ca\(^{2+}\)-dependent down-regulation of NOS mediated by the phosphorylation of NOS protein and the resultant decrease in the activity (30) or the dual regulation of constitutive NOS activity by Ca\(^{2+}\) (31) is not likely, because the Ca\(^{2+}\) concentration they employed (10–2000 \(\mu M\)) exceeded the physiological Ca\(^{2+}\) range. Furthermore, we should be very careful if biochemical study in cell-free system does take place in vivo also.

The Ca\(^{2+}\) reduction in cocultured VSMCs became stable 3 min after ATP or BK stimulation (Fig. 5, 6 and 7), and it was sustained for up to 40 min after TG or IM stimulation. The long lasting action of TG and IM is compatible with a continuous production of NO (Fig. 2), because the biological lifetime of NO is very short (3). In the late phase of TG-induced Ca\(^{2+}\) transients, the low Ca\(^{2+}\) level, which was completely supported by the Ca\(^{2+}\) influx, stimulated ECs to produce large amounts of NO. These results suggest that Ca\(^{2+}\) influx might trigger NO production and that the activation of NOS does not require high concentrations of Ca\(^{2+}\) to sustain the NO production.

NO Production without Agonist—High concentration of L-arginine alone weakly decreased the Ca\(^{2+}\) in ECs and markedly reduced the Ca\(^{2+}\) in cocultured VSMCs. These Ca\(^{2+}\) modulations were mediated by NO, because NO was actually released from ECs and detected in the medium (Fig. 11). Furthermore, D-arginine or L-lysine has no NO producing action (data not shown). Thus, both the Ca\(^{2+}\) handling and NO production are modified specifically by the high concentrated L-arginine, and these phenomena are not due to nonspecific charge effect nor basic amino acid.

In addition, the NO production induced by L-arginine was not dependent on Ca\(^{2+}\) (Fig. 11). The L-arginine study cannot be explained by the Ca\(^{2+}\)-CaM theory and suggests that a basal or even lowered Ca\(^{2+}\) level within ECs is sufficient to activate eNOS, if a high dose of substrate is supplied. No biochemical data are available on the relationship between NOS activation and the substrate concentration. After the NO is produced, the L-arginine concentration might not significantly reduce, because it is supplied from the glutamine or recycled from L-citrulline within a cell (32). However, several clinical reports (33, 34) indicated that L-arginine might potentiate the NO production. Present studies gave direct evidence for that.

Our previous data with the measurement of cGMP (35) have suggested that NO is basically released in vivo without agonist stimulation, just under shear stress. The basal release of NO evident in this study could also be present in vivo (36), because vascular ECs are usually under shear stress. The Ca\(^{2+}\) in ECs is dependent on the blood flow rate (35), which could stimulate basal NO synthesis. The subsequent NO production determined by physiological flow could serve to inhibit the proliferation of ECs (18) or VSMCs (37) and prevent platelet adhesion.

**Fig. 11.** Two-dimensional images of the Ca\(^{2+}\) response of ECs and VSMCs in coculture (×200) induced by L-arginine alone and the Ca\(^{2+}\) dynamics in both cells. The experiments show fura-2-loaded cultured cells in the resting state at an excitation wavelength of 380 nm (A), the F\(_{340}/F\_380\) ratio control image (B), and 360 s (C) after the addition of L-arginine (5 mM). D, the Ca\(^{2+}\) transient in ECs (●) and VSMCs (○). E, NO production in cocultured ECs with (●) or without (△) Ca\(^{2+}\). At the arrow, L-arginine (5 mM) was added. Each point denotes the mean ± S.E. (n = 10–12). The bar represents 100 \(\mu\)m.

**Fig. 12.** Correlation between the HDT of Ca\(^{2+}\) in the degeneration phase and NO production (A), AUC and NO production (B), or the peak Ca\(^{2+}\) level and NO production (C). Each point denotes the mean ± S.E. (n = 12).
(38), suppressing the progression of arteriosclerosis or thrombus formation in the vessel wall.

NO Action on Ca2—Handling in VSMCs—The Ca2 of VSMCs varies to the agonist stimulation, dependent on culture conditions (9, 10). All four agonists studied directly increased Ca2 in isoculture of VSMCs while indirectly decreasing the Ca2 through the action of NO in coculture with ECs. Therefore, the observed Ca2 dynamics of cocultured VSMCs should be viewed as the net difference of the two opposite actions. ATP, BK, and TG that directly raised the Ca2 was counterbalanced by the lowering of the Ca2 by NO, resulting in a net Ca2 reduction (Figs. 6–8). IM induced no net Ca2 reduction in cocultured VSMCs but hampered the Ca2 rise expected in isocultures of VSMCs (Fig. 10), indicating that the direct Ca2 raising action of IM on VSMCs exceeded the Ca2 lowering action of NO present in cocultures with ECs.

NO might have several routes to reduce the Ca2 in VSMCs as follows: (i) NO activates a guanylate cyclase in the soluble fraction of VSMCs and increases the intracellular concentration of cGMP, which accelerates the efflux of Ca2 (39). (ii) NO may directly inhibit Ca2 entry through voltage-dependent Ca2 channels (40) or may enhance the outward K+ current by activating Ca2-dependent K+ channels that cause hyperpolarization (19), and (iii) NO could potentiate the Na+-Ca2 exchange (41).

The exogenous administration of NO gas is impervious to exactly control NO concentration without oxidation to NO2 in the incubation medium. NO donors, such as sodium nitroprusside and s-nitroso-n-acetyl-d,L-penicillamine, are also inadequate for this quantitative purpose because the rate of NO supply is uncertain. This is the first report that for the most part resolves these problems by using NO naturally produced from ECs. By combining NO determination with NO bioassay measuring the Ca2 transients of ECs and VSMCs in coculture, we have succeeded both in identifying the significant component of the Ca2 responsible for NO production in ECs and in quantifying the relationship between NO release and the subsequent Ca2 reduction in VSMCs.

The present methods of coculturing different cell types, of measuring intracellular information by two-dimensional imaging of each cell, and of simultaneously determining released factors are of great value not only in the understanding of vascular biology but also in the examination of the relationships between a wide variety of cell populations that coexist in close proximity and require intracellular communication to regulate their mutual interaction.

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