Autocrine Action and Its Underlying Mechanism of Nitric Oxide on Intracellular Ca\(^{2+}\) Homeostasis in Vascular Endothelial Cells*

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The rise in cytosolic Ca\(^{2+}\) concentration (Ca\(^{2+}\)i) in vascular endothelial cells (ECs) activates the production and release of nitric oxide (NO). NO modifies Ca\(^{2+}\)i homeostasis in many types of nonendothelial cells. However, its effect on endothelial Ca\(^{2+}\)i homeostasis at basal and excited states remains unclear. In the present study, to elucidate the effect of NO on basal Ca\(^{2+}\)i, inositol 1,4,5-trisphosphate-induced Ca\(^{2+}\)i release (IICR) was blocked by expressing an antisense against type-1 inositol 1,4,5-trisphosphate receptors or by microinjecting heparin to individual ECs, and the effects of NO that was released by and diffused from adjacent IICR-intact ECs were recorded. After ATP or bradykinin stimulation, IICR-inhibited ECs showed a marked reduction of basal Ca\(^{2+}\)i, which was abolished by N\(^\ominus\)-monomethyl-L-arginine monoacetate pretreatment. The reduction disappeared in sparsely seeded ECs. Exogenous NO gas mimicked the effect of ATP or bradykinin to reduce basal Ca\(^{2+}\)i. Blocking plasma membrane Ca\(^{2+}\)-ATPase (PMCA), but not Na\(^{+}\)-Ca\(^{2+}\) exchange or sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase, suppressed the reduction, indicating that the reduction resulted from a NO-dependent potentiation of PMCA. To elucidate the effect of NO on elevated Ca\(^{2+}\)i, ATP-, bradykinin-, or thapsigargin-evoked Ca\(^{2+}\)i response in the presence and absence of NO production was compared in adjacent IICR-intact ECs. NO was found to potentiate PMCA, which, in turn, greatly attenuated agonist-evoked Ca\(^{2+}\)i elevation. NO also potentiated Ca\(^{2+}\)i influx, which markedly increased the sustained phase of Ca\(^{2+}\)i elevation and possibly NO production. NO did not affect other Ca\(^{2+}\)i-elevating and Ca\(^{2+}\)i-sequestrating components. Thus, NO-dependent potentiation of PMCA is crucial for Ca\(^{2+}\)i homeostasis over a wide Ca\(^{2+}\)i range.

Vascular endothelial cells (ECs)1 play an important role in the regulation of blood pressure and local blood flow. ECs respond to physical stimuli and blood-borne chemical signals with the production and release of vasoactive substances that directly affect the tone of vascular smooth muscle cells (VSMCs) (1–4). Cytosolic free Ca\(^{2+}\) concentration (Ca\(^{2+}\)i) in ECs plays a crucial role in these processes. For example, Ca\(^{2+}\)i elevation activates endothelial nitric-oxide synthase to produce nitric oxide (NO) (5, 6), which is the most potent substance to decrease VSMC tone and proliferation. In ECs, a typical Ca\(^{2+}\)i elevation evoked by G-protein-coupled receptor agonists consists of an initial spike and a subsequent sustained phase (7–9). The initial spike mainly originates from inositol 1,4,5-trisphosphate (IP\(_3\))-induced Ca\(^{2+}\) release (IICR) from the endoplasmic reticulum, and the following sustained phase results from capacitative Ca\(^{2+}\) entry (CCE) across into the plasma membrane (10, 11). In addition to these Ca\(^{2+}\)i-elevating parts, Ca\(^{2+}\)i is also regulated by Ca\(^{2+}\)i-sequestrating components, including Ca\(^{2+}\)i uptake by sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) (12, 13), Ca\(^{2+}\)i extrusion by plasma membrane Ca\(^{2+}\)-ATPase (PMCA) (14–17), and Na\(^{+}\)-Ca\(^{2+}\) exchange (NCX) (16–18). Our previous studies have shown that IICR is essential for the induction of CCE in ECs and that CCE plays an important role in the continuous production of NO (7, 8). On the other hand, in a series of nonendothelial cell types, NO has been reported to modify almost all Ca\(^{2+}\)i-sequestrating components, including Ca\(^{2+}\)i-elevating parts, Ca\(^{2+}\)i-sequestrating components (19, 20). NO attenuates IICR at several steps, by inhibiting G-protein in VSMCs (21) and platelets (22), by inhibiting phospholipase β in PC12 cells (23), and by inhibiting IP\(_3\) receptors in VSMCs (24). The effects of NO on CCE vary among cell types (19, 20, 25–30); NO potentiates CCE in pancreatic acinar cells (25–27) and colonic epithelial cells (28), does not affect CCE in Jurkat T-lymphocytes (29) and embryonic kidney cells (30), and inhibits CCE in platelets (31) and VSMCs (32). Regarding the effects on Ca\(^{2+}\)i-sequestration, NO potentiates PMCA (33, 34), NCX (35–37), and SERCA (32) in VSMCs, platelets, and astrocytes. However, in ECs, the effect of NO on Ca\(^{2+}\)i homeostasis including Ca\(^{2+}\)i-elevating and Ca\(^{2+}\)i-sequestrating components remains unclear.

On the other hand, most of the published results so far have been obtained by using exogenous NO. The effects on Ca\(^{2+}\)i homeostasis varied among NO donor, NO gas, and endogenous NO, even in the same cell type (19, 20, 34, 38, 39). In the Ca\(^{2+}\)-containing intracellular Ca\(^{2+}\); CCE, capacitative Ca\(^{2+}\) entry; IICR, inositol 1,4,5-trisphosphate-induced Ca\(^{2+}\) release; IM, ionomycin; IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_{R_1}\), type 1 IP\(_3\) receptor; l-NMMA, N\(^\ominus\)-monomethyl-L-arginine monoacetate; NCX, Na\(^{+}\)-Ca\(^{2+}\) exchange; NO, nitric oxide; PMCA, plasma membrane Ca\(^{2+}\)-ATPase; SERCA, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase; TG, thapsigargin; VSMC, vascular smooth muscle cell.
Transient Transfection of ECs—

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...ent ECs. Transfection was done in a serum-free medium using a mix-
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Preparation of Antibodies to Isoforms of IP\(_3\) Receptor and Immunoblotting—Three peptides were synthesized according to the amino acid sequence of the cytosolic C-terminal domain of human type 1 (GHP-PHMNVFQPQ(C)), type 2 (CILGSNTPHVNNHMPPH), and type 3 ((C)RQRLGFVDVQNCISR) IP\(_3\) receptors. All of them were synthesized and puriﬁed on a hydroxyapatite column in 6% gel. Samples were then electrotransferred to nitrocellulose membranes using a semidy blotter. Membranes were incubated with antibodies (\(\times 200\)) against type 1, 2, or 3 IP\(_3\) receptor. Subsequently, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Antigens recognized by antibodies were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Partial Cloning of Bovine IP\(_3\)R Gene, Construction of Vector, and Transient Transfection of ECs—Total RNA from cultured bovine aortic ECs was used as a template to generate first strand cDNA using an oligo(dT)\(_{18}\) primer. A two-strand DNA fragment of the bovine IP\(_3\)R gene was ampliﬁed by polymerase chain reaction from cDNA using primers (upper, 5'-catagcttaaagag-9') and (lower, 5'-catagcttaaagagcgcgtc-3'). The single fragment obtained was inserted into the pCR\(_{TM}\) TA cloning vector (Invitrogen) and sequenced (Hitachi-5500). The fragment spanned 379 bases (–197 to +182) shared 94% homology with human and 93% homology with mouse in the corresponding domain of the IP\(_3\)R gene. The fragment was then cloned into pEGFP\(_C1\) (CLONTECH), and antisense (pG.IP3R\(_R\)-AS) or sense (pG.IP3R\(_S\)-S) orientation of the insert was conﬁrmed by sequencing. To reduce the intercellular distance between transfectedants and adjacent untransfected cells and to decrease the transfection rate, we used 10% confluent ECs. Transfection was done in a serum-free medium using a mixture of 1.33 \(\mu\)g of pG.IP3R\(_S\)-AS and 3.3 \(\mu\)l of Lipofect (Life Technologies, Inc.) per 1.2-mm diameter dish. This method resulted in a low transfection rate of 4–6%. pG.IP3R\(_S\)-S was used as the control. 12–24 h after transfection, the medium was changed to Dulbecco modiﬁed Eagle’s medium with 10% fetal bovine serum. Cells were collected 24 h after the medium was changed.

Effects of NO on Basal Ca\(^{2+}\) i in ICR-inhibited Transfectants—Immunoblotting revealed that the antibody against IP\(_3\)R\(_1\) reacted with a single 260-kDa band in the crude homogenate of cultured bovine aortic ECs (Fig. 1A). In contrast, the injection volume into a cell was controlled by varying heparin (Eppendorf 5171 micromanipulator and 5246 transjector, Hamburg). The injection cell was controlled by varying heparin concentration and injecting pressure (5–80 mmH\(_2\)O). Within 5 min after the injection, the medium was changed to HEPES buffer solution containing 1 mM Ca\(^{2+}\) i and incubated further for 10 min to stabilize the injected cells.

Measurement of Ca\(^{2+}\) i and Mn\(^{2+}\) i influx—ECs were stimulated by ATP, BK, ionomycin (IM), or thapsigargin (TG). All of the four agonists were obtained from Sigma. Ca\(^{2+}\) i responses of transfected and untransfected cells as well as heparin-injected and -uninjected cells in the same observation field were individually analyzed by a two-dimensional Ca\(^{2+}\) imaging system, as reported previously (8, 41). The absolute Ca\(^{2+}\) i was calculated, by comparing the ﬂuorescence ratios at both wavelengths obtained at maximum Ca\(^{2+}\) i, (achieved by lysing the cells and removing fura-2 with Ca\(^{2+}\) i) and minimum Ca\(^{2+}\) i (achieved by chelating all free Ca\(^{2+}\) i with EGTA) using the following equation: Ca\(^{2+}\) i = \(K_0 \times (R - R_{\text{min}})/(R_{\text{max}} - R) \times S_{\text{F2/FS2}} K_0 \) is the dissociation constant (224 nm for fura-2), and \(R_{\text{min}}\) and \(R_{\text{max}}\) are the \(F_{\text{F0}/F_{\text{SO}}}\) ratios of the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms. \(S_{\text{F2/FS2}}\) is the ratio of the ﬂuorescence values at 380-nm excitation determined at \(R_{\text{min}}\) and \(R_{\text{max}}\) respectively. Mn\(^{2+}\) (0.5 mM)-induced quenching of fura-2 ﬂuorescence was recorded at the excitation wavelength of 360 nm (\(F_{\text{FS}}\)) for measurement of the cation influx rate. The percentage Mn\(^{2+}\) quenching was obtained from the dynamic \(F_{\text{SO}}\) divided by the basal \(F_{\text{SO}}\).

Immunoﬂuorescence—CECs grown on CELLlocate coverslips (Eppendorf) were rinsed with phosphate-buffered saline (pH 7.4) (8) and then ﬁxed in the same buffer plus 3.6% paraformaldehyde. All subsequent steps were performed in Ca\(^{2+}\)-free and Mg\(^{2+}\)-free buffer solution. Heparin was added to the injection solution at 1% for 24 h before experiments. Cells of 4–12 passages were used in this study.

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Solutions—HEPES buffer solution, which contained 145 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 10 mM d-glucose, and 10 mM Hepes (pH 7.4), was utilized as the extracellular medium. The Ca\(^{2+}\)-free medium consisted of HEPES buffer solution in which CaCl\(_2\) was replaced with EGTA (1 mM). To block PMCA, we employed a buffer consisting of 115 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 20 mM MgCl\(_2\), 10 mM d-glucose, and 10 mM Hepes (pH 8.8) (33). For blockade of both PMCA and NCX, La\(^{3+}\) (125 \(\mu\)M) was added to the NO buffer.

Cell Microinjection—After loading fura-2/AM (4 \(\mu\)M, Molecular Probes, Inc., Eugene, OR) for 40 min, ECs were rinsed and incubated in the Ca\(^{2+}\)-free medium. Heparin (\(M_0 = 5000\); Wako) dissolved in the vehicle (48 mM K\(_2\)HPO\(_4\), 14 mM Na\(_2\)HPO\(_4\), 4.5 mM KH\(_2\)PO\(_4\), and 400 \(\mu\)M unesteriﬁed fura-2, pH 6.9) was injected into the perinuclear cytoplasm (Eppendorf 5171 micromanipulator and 5246 transjector, Hamburg).

RESULTS

Effects of NO on Basal Ca\(^{2+}\) i in ICR-inhibited Transfectants—Immunoblotting revealed that the antibody against IP\(_3\)R\(_1\) reacted with a single 260-kDa band in the crude homogenate of cultured bovine aortic ECs (Fig. 1A). In contrast,
neither type 2 nor type 3 IP3 receptor was detected. A pharmacological study of Ca2+ dynamics revealed that ECs responded weakly (ΔCa2+i rise = 11 nM) to caffeine (3–60 μM), with or without ryanodine pretreatment (10–60 μM; data not shown). These results suggest that IICR mediated by IP3R1, but not Ca2+-induced Ca2+ release, could be the major mechanism involved in Ca2+ release in ECs.

Of the 4–6% of cells that were successfully transfected with pG.IP3R1-AS, 16% showed reduced IP3R1 expression (data not shown), and 12% showed almost complete inhibition of IP3R1 expression by immunocyto staining (Fig. 1B). On the other hand, the surrounding untransfected cells and cells transfected with pG.IP3R1-S demonstrated normal staining, indicating that the reduction or complete inhibition of IP3R1 expression was a specific effect of pG.IP3R1-AS (Fig. 1, B and C).

Individual transfectants surrounded by untransfected cells were selected to measure Ca2+ handling. Of all of the pG.IP3R1-AS transfectants, about 15 and about 12% demonstrated attenuated and complete inhibition of Ca2+ response to ATP or BK, respectively. The incompletely (data not shown) or completely (Fig. 1B) inhibited Ca2+ response is consistent with immunocyto staining results. Ca2+ dynamics in cells with incomplete IICR inhibition exhibited a delayed and shortened initial Ca2+ spike, with subsequent Ca2+ reduction below the basal level after ATP (ΔCa2+i = 74 ± 5 nM, Fig. 2A) or BK (ΔCa2+i = 70 ± 7 nM) stimulation. Ca2+ dynamics in cells with
Fig. 2. ATP-induced Ca$^{2+}$i dynamics in pG.IP$_{3}$R$_{1}$-AS transfectants in the presence of 1 mM Ca$^{2+}$. Shown are ATP (10 µM)-induced Ca$^{2+}$i dynamics in cells with incomplete (A and B) and complete (C and D) IICR inhibition with (B and D) and without (A and C) pretreatment with L-NMMA (1 mM) for 30 min between two ATP applications (1st and 2nd, arrows). L-NMMA was added to the medium throughout ATP stimulation ($n = 5$–6).

Complete IICR inhibition demonstrated not only no initial Ca$^{2+}$i spike but also marked Ca$^{2+}$i reduction immediately after ATP ($\Delta$Ca$^{2+}$i$_{-}$ = 84 ± 5 nM; Figs. 1B and 2C) or BK ($\Delta$Ca$^{2+}$i$_{-}$ = 73 ± 4 nM; Fig. 1B) stimulation. After a wash-out of the first ATP solution followed by a 30-min equilibration period, these Ca$^{2+}$i reductions were reproducible by secondary ATP stimulation (Fig. 2, A and C). On the other hand, in pG.IP$_{3}$R$_{1}$-S transfectants, ATP and BK induced a normal Ca$^{2+}$i response (Fig. 1C).

Our previous study has shown that NO is produced upon stimulation with ATP or BK, and ATP-induced NO production is reproducible and can be blocked by l-NMMA in bovine aortic ECs (8). Therefore, Ca$^{2+}$i reduction with or without NO production was compared in the same cell. Cells that exhibited marked Ca$^{2+}$i reduction after the first ATP stimulation were pretreated with l-NMMA (1 mM; Calbiochem) for 30 min. This procedure dramatically attenuated Ca$^{2+}$i reduction ($\Delta$Ca$^{2+}$i$_{-}$ = 16 ± 4 nM) at the second ATP stimulation (Figs. 2, B and D), suggesting that Ca$^{2+}$i reduction was caused by endogenous NO. However, Ca$^{2+}$i reduction was unchanged by pretreatment with indomethacin (25 µM) for 30 min (data not shown).

Effects of NO on Basal Ca$^{2+}$i in IICR-inhibited ECs by Microinjection of Heparin—Since IICR inhibition induced by pG.IP$_{3}$R$_{1}$-AS is a relatively slow process, the effects of NO were further investigated in ECs where IICR was directly inhibited by heparin. As shown in Fig. 3, cell 1, injected with 50 mg/ml of heparin at 10 mM H$_{2}$O, exhibited an incomplete inhibition of the initial Ca$^{2+}$i spike, with a subsequent Ca$^{2+}$i reduction below the basal level in response to ATP stimulation. Cell 2 and cell 3, injected with 200 mg/ml of heparin at 20 mM H$_{2}$O, exhibited a complete inhibition of the initial Ca$^{2+}$i spike and, moreover, marked Ca$^{2+}$i reduction ($\Delta$Ca$^{2+}$i$_{-}$ = 103 ± 4 nM), similar to that occurring in pG.IP$_{3}$R$_{1}$-AS transfectants. The reduction of Ca$^{2+}$i in cells with complete IICR inhibition was reproducible in the second ATP stimulation ($\Delta$Ca$^{2+}$i$_{-}$ = 102 ± 4 nM, Fig. 3, B and D). BK also induced Ca$^{2+}$i reduction in IICR-inhibited cells ($\Delta$Ca$^{2+}$i$_{-}$ = 98 ± 6 nM). As shown in Fig. 4A, microinjection of vehicle or heparin (200 mg/ml) had no effect on basal Ca$^{2+}$i during a 60-min follow-up period. Ca$^{2+}$i reduction was induced only by the application of an agonist, and it was unlikely that the nonspecific effects of heparin were involved in the process. Cells that exhibited complete IICR inhibition after the first ATP stimulation were pretreated with l-NMMA for 30 min. This procedure completely abolished the marked Ca$^{2+}$i reduction and led to nearly no Ca$^{2+}$i rise at the second ATP stimulation ($\Delta$Ca$^{2+}$i$_{-}$ = 2 ± 4 nM; Fig. 4B). When heparin was injected into ECs that were plated at a low density, no Ca$^{2+}$i reduction was observed after ATP (Fig. 4C) stimulation. Furthermore, in sparsely seeded ECs, treatment with NO gas solution (3 µM) induced Ca$^{2+}$i reduction in cells injected with heparin ($\Delta$Ca$^{2+}$i$_{-}$ = 38 ± 7 nM) as well as the adjacent IICR-intact cells ($\Delta$Ca$^{2+}$i$_{-}$ = 19 ± 5 nM; Fig. 4D). These findings demonstrated that the reduction of basal Ca$^{2+}$i, caused by NO also occurred in heparin-injected cells.

Changes of Ca$^{2+}$i Influx, Internal Ca$^{2+}$i Stores, and Ca$^{2+}$i Extrusion in IICR-inhibited ECs—The reduction of basal Ca$^{2+}$i was reversible naturally (Fig. 5, A, a). Mn$^{2+}$ quenching showed that ATP-induced (Fig. 5A, c) or BK-induced (data not shown) Ca$^{2+}$i influx was almost absent during the reduction. After removal of the agonist, the reduction was restored relatively rapidly (Fig. 5A, b), during which Ca$^{2+}$i influx increased mildly (Fig. 5A, c).

Next, the Ca$^{2+}$i sequestration pathway through which NO reduced basal Ca$^{2+}$i, was examined. First, we investigated whether the reduction of Ca$^{2+}$i was a result of a NO-dependent potentiation of SERCA. In the absence of Ca$^{2+}$i, ATP- or BK-induced Ca$^{2+}$i release is caused by IICR, and further Ca$^{2+}$i release induced by IM represents the residual content of internal Ca$^{2+}$i stores. In pG.IP$_{3}$R$_{1}$-AS transfectants and cells injected with heparin, no significant increase in IM-releasable stores was found in between cells that showed marked Ca$^{2+}$i reduction after ATP stimulation and cells that were directly stimulated by IM (Fig. 5B). Cells used in the study of direct IM stimulation demonstrated an ATP-induced Ca$^{2+}$i reduction 30 min prior to IM addition. Furthermore, at 20 s after TG addition, ATP (Fig. 5C, a) or BK (data not shown) still induced a reduction of basal Ca$^{2+}$i. The reduction, however, is attenuated compared with the case without TG. In IICR-inhibited cells, TG induced Ca$^{2+}$i elevation in the Ca$^{2+}$i-free medium (data not shown). TG-ATP-induced (Fig. 5C, b) or TG-induced (Fig. 5C, c) Mn$^{2+}$ quenching increased compared with ATP-induced quenching (Fig. 5A, c). Thus, the attenuation of Ca$^{2+}$i reduction might be caused by the counteraction of the reduction by TG-induced Ca$^{2+}$i leakage from ER and TG-induced Ca$^{2+}$i influx. These results proved that Ca$^{2+}$i reduction in IICR-inhibited cells was not caused by SERCA stimulation.

Second, we investigated whether the reduction of basal Ca$^{2+}$i was a result of NO-dependent Ca$^{2+}$i extrusion by PMCA (Na$^{+}$-
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**Fig. 3. Two-dimensional images of Ca²⁺ responses and Ca²⁺ dynamics induced by ATP in heparin-injected ECs in the presence of 1 mM Ca²⁺.** A, cell position and time-serial two-dimensional images of Ca²⁺ responses to first ATP (1st ATP, 10 μM) stimulation. Cell 1 (I), injected with 50 mg/ml heparin, showed a delayed and attenuated Ca²⁺ rise. Cell 2 (II) and cell 3 (III), injected with 200 mg/ml heparin, showed marked Ca²⁺ reduction. Shown is the localization of ECs and Ca²⁺ responses at 10, 20, 35, and 90 s after ATP stimulation. B, fura-2-loaded cells and time-serial images of Ca²⁺ responses to second ATP (2nd ATP, 10 μM) stimulation. Ca²⁺ rise was partially recovered in cell 1 but remained suppressed in cell 2 and cell 3. Shown are images of fura-2-loaded cells and Ca²⁺ responses at 5, 10, 35, and 90 s after ATP stimulation. C and D, Ca²⁺ dynamics of cell 1 and cell 2 after ATP stimulation (arrow), respectively. Ca²⁺ dynamics of cell 3, which is similar to that of cell 2, is omitted (n = 4). Bar, 50 μm.

independent Ca²⁺ efflux) or NCX (Na⁺-dependent Ca²⁺ efflux). Blockade of PMCA by 20 mM extracellular Mg²⁺ (pH 8.8; Fig. 6A) almost completely abolished the NO-induced Ca²⁺ reduction (ΔCa²⁺ᵢ = 82 ± 4 nM; 8 ± 5 nM) in IICR-inhibited cells. Blockade of NCX by replacing extracellular Na⁺ with choline did not affect the reduction (ΔCa²⁺ᵢ = 82 ± 4 nM; 8 ± 6 nM; Fig. 6B). Blockade of both PMCA and NCX by 125 μM La³⁺ and 0 mM Na⁺ also eliminated the reduction (ΔCa²⁺ᵢ = 82 ± 4 nM; 11 ± 6 nM; Fig. 6C). To rule out the possibility that these blockades might decrease NO production in adjacent IICR-intact cells, NO₂⁻ production was assayed. Even with the blockade of PMCA and/or NCX, NO was produced during BK stimulation (Fig. 6D). Thus, Ca²⁺ᵢ reduction in IICR-inhibited cells was caused by the NO-dependent potentiation of Ca²⁺ᵢ efflux by PMCA.

**Effects of NO on Agonist-evoked Ca²⁺, Elevation in Adjacent IICR-intact ECs—**The effects of NO on agonist-evoked Ca²⁺ elevation were investigated by comparing Ca²⁺ᵢ responses in the presence and absence of NO production. Inhibition of NO production by L-NMMA significantly potentiated the initial spike in the presence and absence of 1 mM Ca²⁺ (Figs. 7, A and B, and 8, A and B, and Table I). Our previous study has proven that NO is produced in the Ca²⁺⁻free medium after ATP or BK stimulation, although the amount produced is less than that produced in the presence of 1 mM Ca²⁺ (8).

First, the mechanism of NO-dependent attenuation of the initial Ca²⁺ᵢ spike was investigated. The effect of NO on ATP- or BK-induced IP₃ production was measured by radioimmunoassay. The basal IP₃ concentration was 0.7 ± 0.1 μM in ECs. In the Ca²⁺⁻free medium, ATP- and BK-induced IP₃ concentrations were 2.8 ± 0.3 and 2.9 ± 0.3 at 15 s and 2.7 ± 0.4 and 2.9 ± 0.4 μM at 120 s after stimulation, respectively (n = 4). After L-NMMA pretreatment, ATP- and BK-induced IP₃ concentrations were 2.9 ± 0.4 and 3.1 ± 0.3 at 15 s and 2.8 ± 0.4 and 3.0 ± 0.7 μM at 120 s after stimulation, respectively (n = 4). Therefore, IP₃ concentration was not significantly changed in cells with and without pretreatment with L-NMMA after both ATP and BK stimulus. This indicates that NO did not inhibit IP₃ production in ECs.

Even after the initial Ca²⁺ᵢ spikes, spikes were attenuated by NO in the Ca²⁺⁻free medium, IM-induced residual Ca²⁺ᵢ release was not significantly changed (Fig. 8, Table I). Furthermore, TG (1 μM) was used at 20 s before treatment with ATP or BK in the Ca²⁺⁻free medium. Under the inhibition of SERCA, L-NMMA pretreatment still potentiated the agonist-evoked initial Ca²⁺ᵢ spike (Fig. 9A, Table I), suggesting that NO did not potentiate SERCA.

The following studies were addressed to investigate the NO-dependent potentiation of Ca²⁺ᵢ efflux. In the presence of 1 mM Ca²⁺, the effect of NO on BK-induced Ca²⁺ᵢ dynamics was observed after the blockade of PMCA or NCX, while the SERCA mechanism remained operational. Blockade of PMCA by 20 mM Mg²⁺ (pH 8.8) significantly raised BK-induced Ca²⁺ᵢ elevation, decreased Ca²⁺ᵢ influx, and slightly attenuated NO production (Figs. 6D and 7, B and F, and Table I). Under the blockade of PMCA, L-NMMA pretreatment did not markedly influence these effects (Fig. 7, C and G). Under the blockade of NCX, L-NMMA pretreatment mildly potentiated Ca²⁺ᵢ elevation. Blockade of both PMCA and NCX by 0 mM Na⁺ and 125 μM La³⁺ raised BK-induced Ca²⁺ᵢ elevation, decreased Ca²⁺ᵢ influx, and attenuated NO production (Fig. 7, D and H, Table I). Under the blockade of both PMCA and NCX, L-NMMA pretreatment did not markedly influence these effects. These results indicated that the NO-de-
pended potentiation of PMCA might be involved in Ca\textsuperscript{2+}, extrusion while Ca\textsuperscript{2+}, is elevated by agonist stimulation.

To prove this hypothesis, we investigated the effect of NO after further inhibition of SERCA by TG (1 \textmu M) at 20 s before BK treatment in the Ca\textsuperscript{2+}-free medium. Blockade of PMCA by 20 mM Mg\textsuperscript{2+} (pH 8.8) significantly raised the BK-induced initial Ca\textsuperscript{2+} spike (Fig. 9A, Table I). Under the blockade of PMCA, further inhibition of NO production did not change the potentiation of the BK-induced Ca\textsuperscript{2+} spike. On the other hand, blockade of NCX showed no significant effect on the BK-induced Ca\textsuperscript{2+} spike (Fig. 9B, Table I). Under the blockade of NCX by 0 mM Na\textsuperscript{+}, further inhibition of NO production mildly potentiated the BK-induced Ca\textsuperscript{2+} spike. Blockade of both PMCA and NCX by 0 mM Na\textsuperscript{+} and 125 \mu M La\textsuperscript{3+} markedly raised the Ca\textsuperscript{2+} spike (Fig. 9C). Under the blockade of both PMCA and NCX, \textalpha-L-NMMA pretreatment did not markedly influence these effects. Similar results were obtained by using TG only, to inhibit SERCA and to stimulate Ca\textsuperscript{2+}, rise and NO production in the Ca\textsuperscript{2+}-free medium (Fig. 9, D–F). NO was produced during TG stimulation even in the presence of PMCA and/or NCX blockade (Fig. 10). These results strongly indicate that the attenuation in the initial Ca\textsuperscript{2+} spike is caused by the NO-dependent potentiation of PMCA, which plays an important role in attenuating Ca\textsuperscript{2+}, signal in the excited state.

Second, in contrast to attenuating the initial Ca\textsuperscript{2+}, spike, NO significantly increased the sustained Ca\textsuperscript{2+} elevation during the sustained phase (Fig. 7, A and B, Table I). As shown by Mn\textsuperscript{2+} quenching of fluorescence, NO markedly potentiated Ca\textsuperscript{2+} influx (from 17 ± 3 to 30 ± 3% at 210 s after ATP stimulation, \( p < 0.05 \); from 18 ± 3 to 27 ± 4% at 210 s after BK stimulation, \( p < 0.05 \); Fig. 7, E and F, and Table I). The potentiated Ca\textsuperscript{2+} influx coincided with the sustained phase of Ca\textsuperscript{2+}, transients. These results suggest that endogenous NO maintains Ca\textsuperscript{2+}, elevation during the sustained phase by promoting CCE.
DISCUSSION

The major findings of the present study are as follows. 1) There is marked reduction of basal Ca^{2+}_{i} after ATP or BK stimulation in individual ECs in which IICR and the subsequent CCE are inhibited by expressing an antisense against IP_{3}R_{1} or microinjecting heparin. 2) The reduction of Ca^{2+}_{i}...
FIG. 7. Effects of NO on the sustained phase of Ca$$^{2+}$$, dynamics and Ca$$^{2+}$$ influx with and without blockade of PMCA or NCX in IICR-intact ECs in the presence of 1 mM Ca$$^{2+}$$. The upper panel shows Ca$$^{2+}$$ dynamics in response to ATP (10 μM; A) and BK (500 nM; B–D) with (thin solid line) or without (thick solid line) pretreatment with l-NMMA (1 mM). Coinciding with the upper panel, the lower panel shows Ca$$^{2+}$$ influx in response to ATP (E) and BK (F–H) with (thin solid line) or without (thick solid line) pretreatment with l-NMMA. Furthermore, in B and F, cells were stimulated under the blockade of PMCA by 20 mM Mg$$^{2+}$$ (pH 6.8) with (thick dotted line) or without (thin dotted line) pretreatment with l-NMMA. Mn$$^{2+}$$ (0.5 mM) was simultaneously applied to cells with stimuli (arrows) (n = 6–10).

TABLE I

Modification of intracellular Ca$$^{2+}$$, dynamics of ECs by NO

|                    | Ca$$^{2+}$$ (+)               | Ca$$^{2+}$$ (-)               |
|--------------------|-------------------------------|-------------------------------|
|                    | Peak                          | 210 s                         | Mn$$^{2+}$$-210 s | Peak                          | Peak-IM                  |
| ATP                |                               |                               |                  |                               |                           |
| ctl                | 394 ± 8                       | 278 ± 9                       | 30 ± 3           | 323 ± 11                      | 205 ± 12                  |
| l-NMMA-ctl         | 480 ± 12$$^{b}$$              | 183 ± 11$$^{b}$$              | 17 ± 3$$^{b}$$   | 400 ± 12$$^{b}$$              | 196 ± 14                  |
| BK                 |                               |                               |                  |                               |                           |
| ctl                | 428 ± 9                       | 292 ± 10                      | 27 ± 4           | 360 ± 10                      | 184 ± 13                  |
| l-NMMA-ctl         | 498 ± 10$$^{b}$$              | 210 ± 10$$^{b}$$              | 18 ± 3$$^{b}$$   | 413 ± 13$$^{b}$$              | 178 ± 15                  |
| hiMg               | 550 ± 9$$^{b}$$               | 440 ± 14$$^{b}$$              | 19 ± 3$$^{b}$$   |                               |                           |
| l-NMMA-hiMg        | 564 ± 11$$^{b}$$              | 445 ± 10$$^{b}$$              | 16 ± 4           |                               |                           |
| 0Na                | 414 ± 11                      | 271 ± 11                      | 28 ± 5           |                               |                           |
| l-NMMA-0Na         | 471 ± 10                      | 210 ± 9                       | 17 ± 3           |                               |                           |
| 0Na-La             | 469 ± 9$$^{c,d}$$             | 370 ± 10$$^{c,d}$$            | 14 ± 3$$^{c,d}$$ |                               |                           |
| l-NMMA-0Na-La      | 482 ± 11                      | 390 ± 8$$^{a}$$               | 12 ± 3$$^{a}$$   |                               |                           |

|                    |                               |                               |                  |                               |                           |
| Ca$$^{2+}$$ (-)    |                               |                               |                  |                               |                           |
| ctl                | 213 ± 5                       | 173 ± 6                       | 354 ± 7          | 334 ± 7                       | 218 ± 5                   |
| l-NMMA-ctl         | 253 ± 5$$^{b}$$               | 178 ± 5                       | 354 ± 6$$^{b}$$  | 334 ± 7$$^{b}$$               | 229 ± 7                   |
| hiMg               | 258 ± 4$$^{b}$$               | 210 ± 5$$^{b}$$               | 354 ± 6$$^{b}$$  | 360 ± 6$$^{b}$$               | 229 ± 7                   |
| l-NMMA-hiMg        | 263 ± 4$$^{b}$$               | 202 ± 4$$^{b}$$               | 354 ± 6$$^{b}$$  | 368 ± 8$$^{b}$$               | 226 ± 8                   |
| 0Na                | 235 ± 5$$^{b}$$               | 162 ± 5$$^{b}$$               | 354 ± 6$$^{b}$$  | 312 ± 6$$^{b}$$               | 227 ± 7                   |
| l-NMMA-0Na         | 256 ± 5$$^{b}$$               | 195 ± 4$$^{b}$$               | 354 ± 6$$^{b}$$  | 339 ± 7$$^{b}$$               | 243 ± 5$$^{b}$$           |
| 0Na-La             | 257 ± 6$$^{a,d}$$             | 218 ± 5$$^{a,d}$$             | 354 ± 6$$^{a,d}$$| 336 ± 5$$^{a,d}$$             | 251 ± 5$$^{a,d}$$         |
| l-NMMA-0Na-La      | 267 ± 7$$^{a}$$               | 208 ± 4$$^{a}$$               | 354 ± 6$$^{a}$$  | 351 ± 4$$^{a}$$               | 250 ± 5$$^{a}$$           |

|                    |                               |                               |                  |                               |                           |
|                   |                               |                               |                  |                               |                           |

<sup>a</sup> ctl, control.
<sup>b</sup> Statistical significance from ctl value at p < 0.05.
<sup>c</sup> Statistical significance from ctl-l-NMMA value at p < 0.05.
<sup>d</sup> Statistical significance from 0Na value at p < 0.05.
<sup>e</sup> Statistical significance from 0Na-l-NMMA value at p < 0.05.

results from NO that was released by and diffused from adjacent IICR-intact ECs. 3) NO reduces basal Ca$$^{2+}$$, by potentiating Ca$$^{2+}$$, extrusion by PMCA. 4) This potentiation also markedly attenuates agonist-evoked Ca$$^{2+}$$, elevation in adjacent IICR-intact cells. 5) Endogenous NO markedly promotes agonist-evoked CCE, which maintains Ca$$^{2+}$$, elevation and possibly NO production in ECs.

Crucial Role of NO-dependent Potentiation of PMCA in Basal Ca$$^{2+}$$, Homeostasis—NO donor or NO gas has been widely used to mimic the effect of endogenous NO (19, 20). However, their effect on basal Ca$$^{2+}$$, has been controversial because of conflicting results. In platelets, Johansson et al. (34) reported that sodium nitroprusside (SNP; 20 μM) reduces basal Ca$$^{2+}$$, by 20–23 nM. In contrast, in ECs, Volk et al. (38) reported that SNP (10–100 μM) significantly increases Ca$$^{2+}$$, up to about 200% of the basal level (ΔCa$$^{2+}$$, = 300 nM), similar to the Ca$$^{2+}$$,
rise induced by 10 μM ATP. Actually, our group also confirmed that 100–300 μM SNP, and not 1–30 μM SNP, mildly increases basal Ca\(^{2+}\); by 10–20 nM in both ECs and VSMCs. However, its nonspecific Ca\(^{2+}\)-raising effect, which is produced by its intermediate or metabolized compounds, could not be excluded from the results, since the Ca\(^{2+}\) rise could not be inhibited by hemoglobin (50 μM).\(^2\) In platelets, Sang et al. (39) reported that a high (5 μM) and not a low concentration of NO gas solution (0.1 μM) increases basal Ca\(^{2+}\) by 10 nM.

On the other hand, our previous studies have shown that after ATP stimulation, endogenous NO that is released by and diffused from ECs decreases basal Ca\(^{2+}\), in adjacent VSMCs by about 40–90 nM in the coculture of both cells (8, 41). The results are consistent with the physiological effect of NO on VSMCs. However, a similar analysis of the effect of endogenous NO on basal Ca\(^{2+}\) in ECs is difficult because agonist-evoked Ca\(^{2+}\) elevation is needed for NO production. Although antagonists against receptor (45), G-protein (46), or phospholipase β (47) are able to inhibit Ca\(^{2+}\) elevation, elevation, they are inadequate for the analysis, since these treatments in a culture dish inhibited Ca\(^{2+}\) elevation in all ECs and consequently led to a decrease in NO production. The effect of NO may only be analyzed in individual ECs in which agonist-evoked Ca\(^{2+}\) elevation is inhibited. In the present study, by using individual ECs in which agonist-evoked Ca\(^{2+}\) elevation was inhibited by the antisense or heparin and by using adjacent excited ECs as a source of NO production, the potent effect of endogenous NO on reducing basal Ca\(^{2+}\) was successfully elucidated. The reduction of basal Ca\(^{2+}\), by endogenous NO has been substantiated, including the partial mimicry by exogenous NO gas. Our studies suggested that endogenous NO is the most ideal source for exploring the physiological effect of NO. NO donor or even NO gas does not always act in the same way as endogenous NO because of difficulty in maintaining the narrow range of effective concentration and mimicking the diffusion pattern of NO in vivo.

The mechanism involved in the reduction of basal Ca\(^{2+}\), was clarified. Our results excluded the involvement of NO-dependent potentiation of SERCA, since there was no consequent increase in IM-releasable Ca\(^{2+}\), stores after the reduction of basal Ca\(^{2+}\) (Fig. 5B), and ATP or BK still induced Ca\(^{2+}\), reduction after SERCA was blocked (Fig. 5C). After blocking SERCA, the attenuation of Ca\(^{2+}\), reduction could be explained by the counteraction by TG-induced Ca\(^{2+}\) leakage from internal stores and TG-induced CCE. The remaining hypothesis is that NO potentiates Ca\(^{2+}\), extrusion by PMCA or NCX. Recently, there is increasing evidence that PMCA is important for Ca\(^{2+}\), extrusion, while NCX plays a minor role (14–18). Thus, the effects of blocking PMCA or NCX on Ca\(^{2+}\), reduction in IICR-inhibited cells were investigated. It was reported that extracellular increase in pH to 8.8 blocks the activity of PMCA in squid axons and red cells, does not alter the activity of NCX and SERCA, and minimally changes intracellular pH (33, 35, 48, 49). Increasing extracellular Mg\(^{2+}\) concentration to 20–30 mM can also block the activity of PMCA in red cells and VSMCs (33, 35, 49). The combination of 20 mM Mg\(^{2+}\) and pH 8.8 blocks PMCA-mediated Ca\(^{2+}\), efflux by up to 80% in VSMCs, whereas it did not affect NCX (33, 35, 50). That PMCA can be blocked by 20 mM Mg\(^{2+}\) (pH 8.8) from the side opposite to the ATP-binding site of the membrane holds true not only for the case in VSMCs but is a general property of the membrane Ca\(^{2+}\) pump ATPase (33, 35, 48–51). Furthermore, extracellular La\(^{3+}\) at concentrations of 60–250 μM specifically blocks PMCA but spares NCX in red cells and VSMCs (IC\(_{50}\) = 50–65 μM; Refs. 33 and 52). Low concentrations of La\(^{3+}\) (20–100 μM) inhibit PMCA activity by displacing Mg\(^{2+}\) from the site at which it combines to accelerate dephosphorylation (53). Therefore, these methods were used to block PMCA of ECs in the present study. On the other hand, NCX was blocked by a Na\(^{+}\)-free buffer. The extracellular Na\(^{+}\)-free buffer would induce changes in intracellular conditions, which, in turn, could affect the activity of PMCA indirectly. However, a previous study showed that the extracellular Na\(^{+}\)-free buffer caused a decrease in intracellular Na\(^{+}\) concentration by 30% in VSMCs (33). Such a decrease in Na\(^{+}\) concentration did not affect the activity of PMCA significantly, since intracellular Na\(^{+}\) and K\(^{+}\) substituted for each other in activating PMCA (54). Several studies have revealed the function of PMCA under the inhibition of NCX using the same method (32, 33, 35, 48–51).

Consequently, Ca\(^{2+}\), reduction was suppressed after the blockade of PMCA but still occurred after the blockade of NCX in IICR-inhibited cells (Fig. 6). These blockades did not significantly affect BK-induced NO production by adjacent IICR-intact cells. Thus, Ca\(^{2+}\), reduction results from the NO-dependent potentiation of Ca\(^{2+}\), extrusion by PMCA, which may be a major and potent regulator of basal Ca\(^{2+}\), homeostasis in ECs. Our results are consistent with previous reports for VSMCs and platelets. In VSMCs, Furukawa et al. (33) reported that SNP or 8-Br-cGMP markedly accelerated the Ca\(^{2+}\), extrusion by PMCA, especially at basal Ca\(^{2+}\), level. In platelets, Johansson et al. (34) reported that both SNP (10 μM) and dibutyryl-cGMP (1 mM) significantly increased V\(_{m}\) of PMCA without affecting its K\(_{m}\) or Hill coefficient. SNP or dibutyryl-cGMP, however, did not change the rate of NCX that has a minor contribution to basal Ca\(^{2+}\), extrusion. As a result of potentiation of PMCA, SNP or dibutyryl-cGMP decreased the basal Ca\(^{2+}\), and attenuated the ionomycin-induced Ca\(^{2+}\), elevation. These effects resulted from a cGMP-induced phosphorylation of PMCA (34).
Ca\(^{2+}\) elevation, consistent with the results of previous studies (16, 55, 56). The present study also showed the significant potentiation of CCE by NO. The lifetime of NO spans only about 6 s (57), suggesting that the potentiation was not due to the NO produced during initial Ca\(^{2+}\) spike but rather to the NO produced during the sustained Ca\(^{2+}\) phase. As shown in Fig. 7, the potentiation is necessary for the maintenance of Ca\(^{2+}\) elevation during the sustained phase, while IICR-intact PMCA and NCX by 125 mM Na\(^{+}\) (C and F). In the upper panel, cells were treated with TG (1 \(\mu\)M) at 20 s before BK (500 nM) stimulation (\(n = 6–10\)).

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Autocrine Action and Its Underlying Mechanism of Nitric Oxide on Intracellular Ca\textsuperscript{2+} Homeostasis in Vascular Endothelial Cells

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