Caveolin is known to down-regulate both neuronal (nNOS) and endothelial nitric-oxide synthase (eNOS). In the present study, direct interactions of recombinant caveolin-1 with both the oxygenase and reductase domains of nNOS were demonstrated using in vitro binding assays. To elucidate the mechanism of nNOS regulation by caveolin, we examined the effects of a caveolin-1 scaffolding domain peptide (CaV1p1; residues 82–101) on the catalytic activities of wild-type and mutant nNOSs. CaV1p1 inhibited NO formation activity and NADPH oxidation of wild-type nNOS in a dose-dependent manner with an IC50 value of 1.8 μM. Mutations of Phe884 and Trp887 within a caveolin binding consensus motif of the oxygenase domain did not result in the loss of CaV1p1 inhibition, indicating that an alternate region of nNOS mediates inhibition by caveolin. The addition of CaV1p1 also inhibited more than 90% of the cytochrome c reductase activity in the isolated reductase domain with or without the calmodulin (CaM) binding site, whereas CaV1p1 inhibited ferricyanide reductase activity by only 50%. These results suggest that there are significant differences in the mechanism of inhibition by caveolin for nNOS as compared with those previously reported for eNOS. Further analysis of the interaction through the use of several reductase domain deletion mutants revealed that the FMN domain was essential for successful interaction between caveolin-1 and nNOS reductase. We also examined the effects of CaV1p1 on an autoinhibitory domain deletion mutant (Δ40) and a C-terminal truncation mutant (ΔC33), both of which are able to form NO in the absence of CaM, unlike the wild-type enzyme. Interestingly, CaV1p1 inhibited CaM-dependent, but not CaM-independent, NO formation activities of both Δ40 and ΔC33, suggesting that CaV1p1 inhibits interdomain electron transfer induced by CaM from the reductase domain to the oxygenase domain.

Nitric-oxide synthase (NOS) generates the physiologically important nitric oxide molecule (NO) from L-arginine (L-Arg) (1–5).
Caveolin-1-interacting Sites in nNOS

Fig. 1. Domain structures of nNOS mutants examined in this study. F584L and W587L harbor mutations in a putative caveolin-1 binding motif within the oxygenase domain. 40 amino acids of the autoinhibitory domain within the FMN subdomain are deleted in the Δ40 mutant, and 33 amino acids from the C terminus are deleted in the ΔC33 mutant. OxCaM is an isolated oxygenase domain containing the CaM binding site. RedCaM and Red are isolated reductase domains with- and without the CaM binding domain, respectively. In the two C-terminal deletion mutants, RedΔNADPH contains amino acids 746–1240, with deletion of the NADPH binding domain, and RedΔFAD/NADPH contains amino acids 746–1169, with deletion of the NADPH and FAD binding domains. RedΔFMN contains amino acids 1170–1429, with deletion of the FMN binding domain.

Accordingly, to examine the interaction between nNOS and caveolin, we developed a rat caveolin-1 expression system in Escherichia coli cells and examined the effects of caveolin-1 protein on nNOS catalysis. Our results indicate that there is a direct interaction of caveolin-1 with both the isolated oxygenase and reductase domains. For further analysis of how caveolin affects NO formation and the electron transfer reactions of nNOS, and to examine which domains of nNOS interact with caveolin, we prepared and analyzed the following nNOS mutants: full-length F584L and W587L (point mutations in the predicted caveolin-1 binding motif of the oxygenase domain), an autoinhibitory insert deletion mutant (Δ40), and a C-terminal truncation mutant (ΔC33: a deletion of 33-amino acids from the C terminus). We also prepared four isolated reductase domain constructs: with (RedCaM) or without (Red) the CaM binding site, the FMN domain only (RedΔNADPH), the FMN/FAD domain (RedΔNADPH), and the FAD/NADPH domain (RedΔFMN) (Fig. 1). In addition, we addressed the effects of caveolin-1 and a scaffolding domain peptide of caveolin-1 (CaV1p1; residues 82–156) on interactions with nNOSs, CaM-dependent or -independent NO formation, and inter- and intradomain electron transfer in these nNOS proteins. We additionally examined the effect of caveolin-3 for reference.

EXPERIMENTAL PROCEDURES

Materials—H, B was purchased from Schircks Laboratories (Jona, Switzerland). 2',5'-ADP-Sepharose and CaM-Sepharose were products of Amersham Biosciences (Uppsala, Sweden). Other reagents were obtained from Sigma or Wako Pure Chemicals (Osaka, Japan). Peptides corresponding to caveolin-1 residues 82–101 (DGIVKASFTTFTVTRY-WFYR termed CaV1p1), 135–156 (DLPLFEAIGSKINRINTQKEI), and caveolin-3 residues 109–130 (KSYLIEIQCISHIYSLCIRTFC) and residues 65–84 (DGVWRVSYTFTVSKYWCYR) were synthesized by Sawady Technology (Tokyo, Japan) and purified by high pressure liquid
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leupeptin, 10 mM full-length nNOS, OxCaM, or Red in a buffer containing 50 mM sodium phosphate (pH 7.5), protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A), and 0.1% Tween 20. The protein mixture was incubated with Ni²⁺-NTA-agarose beads in the same buffer at 4 °C for 60 min, and then the beads were washed three times with the above buffer containing 100 mM imidazole. The bound proteins were eluted with buffer containing 200 mM imidazole.

In some experiments, we used GST-caveolin-1 fusion proteins (5 μM) with OxCaM, His-tagged Red, Red440, Red4FMN, Red4NADPH, or Red4FAD/NADPH protein (5 μM) under the same conditions as mentioned above for the binding assay, with the exception that proteins were adsorbed to glutathione-Sepharose beads instead of Ni²⁺-NTA-agarose beads. These bound proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM glutathione.

In all the cases, the eluted proteins were subjected to 7.5% SDS-PAGE followed by Western blot analysis with polyclonal anti-nNOS N- and C-terminal antibodies (Santa Cruz Biotechnology) or monoclonal anti-His tag antibodies. A horseradish peroxidase-conjugated secondary antibody was used to visualize bound primary antibodies by enhanced chemiluminescence.

To examine effect of CaM on the interaction between caveolin-1 and nNOS, CaM (0, 25, 50, or 250 μM), with 1 mM CaCl₂ was added to the binding assay mixture after a 1-h incubation of caveolin-1 and nNOS. The interaction of caveolin-1 with nNOS and the effect of CaM on this interaction was also examined using a 27-MHz quartz crystal microbalance (QCM) (Initium Co., Tokyo, Japan). Anti-His tag antibodies were immobilized onto a QCM electrode as described by the manufacturer's protocol. The electrode was washed with 50 mM Tris-HCl (pH 7.5) buffer several times for removal of excess antibody, and then soaked in 50 mM Tris-HCl (pH 7.5) buffer (8 ml), and monitored continuously for QCM frequency change at 25 °C. Recombinant caveolin-1 (500 nM) was added to the solution. After the frequency change was stabilized, the buffer was exchanged (8 ml) to remove the excess caveolin-1. 100 nM of bovine serum albumin and then full-length nNOS (100 nM) were stepwise added to the electrode. Finally, the time course of frequency change in response to the addition of CaM (700 nM) and CaCl₂ (1 mM) was assessed.

NO Synthase Activity—NO concentrations for activity measurements were fluorometrically determined as NO₃⁻ by using the NO/NO₃ assay kit (F2, 2,3-Diaminonaphthahaline Kit; Dijindo, Kumamoto, Japan). Unless otherwise indicated, catalytic assays were carried out at 25 °C in a reaction mixture of 50 mM Tris-HCl (pH 7.5), 0.1 mM KCl, 0.1 μM CaM, 100 μM FAD, 5 μM FMN, 5 μM NADPH, and 50 μM dithiothreitol; 0.1 mM L-Arg, 100 units/ml catalase, 10 units/ml superoxide dismutase, 0.1 mM NADPH, 5% MeSO, and 0.1 μM nNOS. To examine the effects of caveolin proteins or peptides, the NOS protein was preincubated for 5 min at 25 °C with various amounts of caveolin proteins or peptides, and then CaM was added to the reaction mixture and incubated for 5 min at 25 °C prior to reaction initiation by addition of NADPH. Initial experiments showed this incubation time was sufficient to reach equilibrium regarding the inhibition.

NADPH Oxidase Activity—The NADPH oxidation rate at 25 °C was monitored by the change of absorbance at 340 nm, using an extinction coefficient of 6.22 nm⁻¹ cm⁻¹. The reaction solution had the same composition as that for the NO formation activity.

Cytochrome c and Ferricyanide Reductase Activities—The cytochrome c and ferricyanide reductase activities were determined by monitoring the change of absorbance at 550 nm or 420 nm, using extinction coefficients of 21 nm⁻¹ cm⁻¹ and 1.01 nm⁻¹ cm⁻¹, respectively. The reaction solution had the same composition as that for NADPH oxidase activity, except for the presence of 0.1 mM cytochrome c or potassium ferricyanide.

RESULTS

Effects of Full-length Caveolin-1 on the NO Formation Activity of Wild-type nNOS—It is known that the peptides of caveolin-1 and -3 scaffolding domains inhibit NO formation by both nNOS and eNOS. To study the mechanism of this inhibition, we expressed caveolin-1 and -3 as His-tagged or GST fusion proteins in E. coli cells, which were purified as described above. The GST-caveolin proteins were exchanged onto a glutathione-Sepharose 4B column, as described by Frangioni and Neel (38).

Interactions of Recombinant Caveolin-1 and Caveolin-3 with nNOS Proteins in Vitro—For the binding assay, purified His-tagged caveolin-1 or caveolin-3 (5 μM) was incubated at 4 °C overnight with 5 μM of full-length nNOS, OxCaM, or Red in a buffer containing 50 mM sodium phosphate (pH 7.5), protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A), and 0.1% Tween 20. The protein mixture was incubated with Ni²⁺-NTA-agarose beads in the same buffer at 4 °C for 60 min, and then the beads were washed three times
lin, perhaps via nonspecific protein-protein interactions. The activities monitored by the fluorescence method were lower than those previously observed by the oxyhemoglobin method, likely due to the longer incubation time (20 min) that is required for NO detection by our fluorescence method. Full-length wild-type nNOS had no NO formation activity in the absence of Ca$^{2+}$/CaM, so the activity was examined in the presence of 0.1 mM CaM and 1 mM Ca$^{2+}$ with different amounts of the recombinant caveolin-1. Under these conditions, we observed inhibition of NO formation by caveolin-1 in the presence of n-octyl-$\beta$-D-glucose (OG); in the absence of OG, the inhibition was not significant due to the aggregation of caveolin-1 (Fig. 2). Similar results were obtained in the presence of Triton X-100 or Tween 20 (data not shown). Addition of the same amount of bovine serum albumin to the system did not affect NO formation. We also examined the effect of recombinant caveolin-3, which was not significant.

The in Vitro Interaction of Caveolin-1 with nNOS—To demonstrate the direct interaction of caveolin-1 with nNOS, we performed in vitro binding assays using full-length caveolin-1 and each of full-length nNOS, the isolated nNOS oxygenase domain containing the CaM binding site (OxCaM), and the isolated nNOS reductase domain (Red). One of the purified nNOS proteins was incubated with or without His-tagged caveolin-1 in the presence of Ni$^{2+}$/NTA agarose beads. As shown in Fig. 3A, an antibody against the C-terminal portion of nNOS or with an antibody against the N-terminal portion of nNOS detected full-length nNOS and Red in fractions incubated with His-caveolin-1 but not in fractions containing nNOS alone. Similarly, an antibody against the N-terminal portion of nNOS detected an interaction between caveolin-1 and the isolated oxygenase domain. These findings suggest that caveolin-1 binds to full-length nNOS and both the isolated oxygenase domain and the isolated reductase domain. Similar direct interactions were observed between His-tagged caveolin-3 and nNOS as shown in Fig. 3B. The interaction of caveolin-1 with either OxCaM or Red was competed by addition of a 4-fold excess of a scaffolding domain peptide (CaV1p1) corresponding to residues 82–101 of caveolin-1 (Fig. 3C). This result suggests that the CaV1p1 sequences interact independently with both the oxygenase domain and the reductase domain of nNOS. Notably, the addition of 5- or 10-fold CaM did not affect the interaction of caveolin-1 with nNOS (Fig. 4A), although addition of a 50-fold excess of CaM did reduce the interaction to a degree. This indicates that CaM only partially competes with caveolin-1 binding to nNOS.

We further examined the interaction of caveolin-1 with nNOS and the effect of CaM using a 27-MHz quartz-crystal microbalance (QCM). QCM is a very sensitive device for the detection of protein-protein interactions in solution, which are
monitored by the linear decrease of the emitted frequency with increasing mass present on the QCM electrode. Steps 1 and 2 in Fig. 4B show the effect of bovine serum albumin and full-length nNOS, respectively, on caveolin-1 immobilized on the QCM electrode. Addition of nNOS decreased the frequency, while bovine serum albumin did not, indicating that there was a specific interaction between nNOS and immobilized caveolin-1. It should be noted that addition of excess CaM significantly, but not completely, released the interaction as shown in Step 3 (Fig. 4B). These results provide further evidence that the interaction between caveolin-1 and nNOS is specific and partially blocked by CaM.

**Effects of Caveolin Peptides on CaM-dependent NO Formation and NADPH Oxidation by nNOS**—Next, we examined NO synthesis catalyzed by full-length nNOS in the presence of two caveolin-1 peptides, a CaV1p1 peptide (residues 82–101) and a peptide containing C-terminal cytoplasmic tail (residues 135–156), and also two of the corresponding peptides of caveolin-3 (residues 65–84 and 109–130). Consistent with previous reports (19), all of the tested peptides inhibited NO formation catalyzed by nNOS in a concentration-dependent manner. In contrast, the nonspecific peptides (PPL)₅ and apamin (CNCKAPETALCARRCQQH) did not affect this activity (data not shown), indicating that the inhibition of NO formation was specific to the caveolin peptides. Among the four tested caveolin peptides, CaV1p1 was the most effective inhibitor, and was thus used for further analysis. CaV1p1 inhibited both NO formation and NADPH oxidation in a dose-dependent manner with an IC₅₀ of 1.8 μM (Fig. 5A and Table I). To study the mechanism of inhibition, a series of enzyme assays were performed in the presence of different concentrations of Ca²⁺/CaM and CaV1p1. In the absence of CaV1p1, CaM activated NO formation with an EC₅₀ value (the concentration of CaM required to induce 50% of the maximal NO formation activity) of 0.13 μM for the wild-type enzyme. Addition of an excess amount of CaM only partially recovered the activity of the CaV1p1-containing systems, depending on the CaV1p1 concentration (Fig. 5B). Double reciprocal plots of NO formation activity versus CaM concentration in the presence of various amounts of the peptide demonstrated a mixed-type inhibition for CaM-dependent NO formation by CaV1p1 (Fig. 5C). This result suggests that CaV1p1 could be both competitive and noncompetitive with CaM for NO formation catalyzed by nNOS. To elucidate the role of the putative caveolin-1 binding motif in the oxygenase domain of nNOS, Phe⁵⁸⁴ and Trp⁵⁸⁷ were mutated to Leu. The optical absorption spectra of each mutant and the CaM, respectively. The NADPH oxidation activities of both mutants were 58 and 28% that of the wild-type enzyme (data not shown). In the absence of CaV1p1, the NO formation activities of the F584L and W587L mutants were 58 and 28% that of the wild-type enzyme, respectively (Table I). Addition of CaV1p1 to the mutants inhibited NO formation activity with IC₅₀ values of 3.5 and 3.0 μM, respectively. The NADPH oxidation activities of both mutants were also inhibited in a dose-dependent manner, similar to the observed inhibition of NO formation. These results indicate that the mutations in the putative caveolin-1 binding motif affected the interaction between nNOS and CaV1p1 in terms of the NO formation and NADPH oxidation rates, but did not totally eliminate the inhibition effect.
Effects of CaV1p1 on Reductase Activities—Ghosh et al. (31) reported that in addition to inhibiting the NO formation activity of eNOS, caveolin-1 peptide also inhibited its cytochrome c reductase activity. To assess whether CaV1p1 inhibits the reductase activity of nNOS as well, we examined the cytochrome c and ferricyanide reductase activities of the various nNOS mutants. CaV1p1 inhibited the cytochrome c reductase activity of wild-type full-length nNOS in a concentration-dependent manner in the presence of Ca²⁺/H11001/CaM (Fig. 6A).

We also examined the isolated reductase domains with (RedCaM) and without (Red) the CaM binding site sequences. The cytochrome c reductase activities of both enzymes were almost completely inhibited by CaV1p1, indicating that the oxygenase domain and CaM binding site are not essential for the inhibition of cytochrome c reductase activity by CaV1p1.

In contrast to cytochrome c reduction, CaV1p1 inhibited ferricyanide reduction by less than 50% as compared with the wild-type enzyme (Fig. 6B). A similar result was obtained for RedCaM. It is known that cytochrome c receives electrons mainly from FMN, whereas ferricyanide can receive electrons from both FMN and FAD in the reductase domain. Therefore, these data suggest that CaV1p1 inhibits the electron transfers between FMN and FAD or between FMN and cytochrome c, but not between NADPH and FAD or between FAD and ferricyanide.

### Table I

| NO formation activity | NADPH oxidation | 
|-----------------------|------------------|
|                        |                  |
| Wild-type 14.1 ± 0.9   | 65 ± 11          |
| F584L 8.2 ± 0.9        | 36 ± 5           |
| W587L 4.0 ± 0.6        | 18 ± 1           |

| **CaV** | **IC₅₀** | **CaV** | **IC₅₀** |
|---------|----------|---------|----------|
| CaV     | 3.4 ± 0.4 | 1.5 ± 0.5 |
| CaV     | 4.3 ± 0.1 | 3.0     |

*Addition of 5 μM CaV1p1 peptide.

*The concentration of CaV1p1 peptide required to induce 50% inhibition of activity.

*The parentheses contain the percent residual activity in the presence of CaV1p1 relative to that in the absence of CaV1p1.

### Fig. 6

**A**, effect of CaV1p1 peptide on cytochrome c reductase activity of full-length wild-type nNOS (circles) or RedCaM (squares) in the presence of 0.1 μM CaM. The effect of CaV1p1 on the activity of Red (triangles) was also examined in the absence of CaM. **B**, effect of CaV1p1 on ferricyanide (filled circles) and cytochrome c (open circles) reductase activities of full-length wild-type nNOS in the presence of 0.1 μM CaM. C, effects of CaM concentration on the inhibition of cytochrome c (white bar) and ferricyanide (striped bar) reductase activities in the wild-type enzyme. Different amounts of CaM were added to the reaction mixture in the presence of 5 μM of CaV1p1 and the reaction was initiated by addition of NADPH after incubation for 5 min at 25 °C.

Effects of CaV1p1 on Reductase Activities—Ghosh et al. (31) reported that in addition to inhibiting the NO formation activity of eNOS, caveolin-1 peptide also inhibited its cytochrome c reductase activity. To assess whether CaV1p1 inhibits the reductase activity of nNOS as well, we examined the cytochrome c and ferricyanide reductase activities of the various nNOS mutants. CaV1p1 inhibited the cytochrome c reductase activity of wild-type full-length nNOS in a concentration-dependent manner in the presence of Ca²⁺/CaM (Fig. 6A). We also examined the isolated reductase domains with (RedCaM) and without (Red) the CaM binding site sequences. The cytochrome c reductase activities of both enzymes were almost completely inhibited by CaV1p1, indicating that the oxygenase domain and CaM binding site are not essential for the inhibition of cytochrome c reductase activity by CaV1p1.

In contrast to cytochrome c reduction, CaV1p1 inhibited ferricyanide reduction by less than 50% as compared with the wild-type enzyme (Fig. 6B). A similar result was obtained for RedCaM. It is known that cytochrome c receives electrons mainly from FMN, whereas ferricyanide can receive electrons from both FMN and FAD in the reductase domain. Therefore, these data suggest that CaV1p1 inhibits the electron transfers between FMN and FAD or between FMN and cytochrome c, but not between NADPH and FAD or between FAD and ferricyanide.
wild-type enzyme in the presence of Ca\(^{2+}\) of the nNOS reductase domain. As shown Fig. 7, the reductase assays using GST-caveolin-1 and His-tagged deletion mutants of excess CaM, suggesting that CaM could reverse only the nNOS Reductase Domain above. To further elucidate the interacting site of caveolin-1 inhibits the electron transfer from FAD to FMN as described CaV1p1 on the reductase domain suggested that caveolin-1 elements in the reductase domain: a 40 amino acid auto-inhibitory loop within the FMN binding subdomain, and a C-terminal sequence of 20–40 amino acids. Deletion of either element affects the Ca\(^{2+}\)/CaM sensitivity of nNOS and eNOS and allows electron transfers to proceed even in the absence of CaM (11–17). To investigate how caveolin binding regulates the FMN subdomain is necessary and sufficient for interaction with caveolin-1.

### Identification of the Interacting Site of Caveolin-1 in the nNOS Reductase Domain

Our observations on the effect of CaV1p1 on the reductase domain suggested that caveolin-1 inhibits the electron transfer from FAD to FMN as described above. To further elucidate the interacting site of caveolin-1 within the reductase domain, we performed in vitro binding assays using GST-caveolin-1 and His-tagged deletion mutants of the nNOS reductase domain. As shown Fig. 7, the reductase domain mutants, Red, Red\(\Delta\)NADPH (the FMN/FAD domain), and Red\(\Delta\)FAD/NADPH (the FMN domain only) interacted similarly with caveolin-1, while the reductase domain without FMN domain (Red\(\Delta\)FMN) did not. These results indicate that the FMN subdomain is necessary and sufficient for interaction with caveolin-1.

### Inhibition of NO Formation Activities of \(\Delta40\) and \(\DeltaC33\) Mutants by CaV1p1—Electron transfers required for the catalytic activity of nNOS and eNOS are also regulated by two control elements in the reductase domain: a 40–46 amino acid auto-inhibitory loop within the FMN binding subdomain, and a C-terminal sequence of 20–40 amino acids. Deletion of either element affects the Ca\(^{2+}\)/CaM sensitivity of nNOS and eNOS and allows electron transfers to proceed even in the absence of CaM (11–17). To investigate how caveolin binding regulates these CaM-independent electron transfers, we examined two deletion mutants of nNOS: \(\Delta40\), in which 40 amino acids have been deleted from the FMN binding subdomain and \(\DeltaC33\), in which 33 amino acids have been truncated from the C-terminal end (Fig. 1).

\(\Delta40\) and \(\DeltaC33\) retained 75 and 60% of the activity of the wild-type enzyme in the presence of Ca\(^{2+}\)/CaM, respectively (Table II). These Ca\(^{2+}\)/CaM-induced NO formation activities were inhibited by addition of CaV1p1 in a concentration-dependent manner as seen in the wild-type enzyme, but with much less sensitivity (Fig. 8). The IC\(_{50}\) values for CaV1p1 with the \(\Delta40\) and \(\DeltaC33\) mutants were 7.0 and 4.5 \(\mu M\), respectively, in the presence of 0.1 \(\mu M\) of CaM. While the EC\(_{50}\) value for CaM with wild-type nNOS was 0.13 \(\mu M\), those for the \(\Delta40\) and \(\DeltaC33\) mutants were 0.07 and 0.05 \(\mu M\), respectively, suggesting that both mutations increased the CaM sensitivity of the enzyme. Therefore, we also examined the CaV1p1-dependent inhibition of the mutants in the presence of 0.05 \(\mu M\) CaM. As shown in Fig. 8 (filled marks), the NO formation activities of both mutants remained less sensitive to inhibition by CaV1p1 even in the presence of low concentrations of CaM. These data suggest that these control elements (the autoinhibitory loop and the C-terminal sequence) are important for the inhibition of CaM-dependent activity of nNOS by CaV1p1, but are not essential for CaV1p1 binding. The NADPH oxidation activities of each mutant were also reduced by CaV1p1, showing good correlation with the inhibition of NO formation in the presence of CaM (Table II). These inhibitory effects were only partially recovered by addition of excess amounts of CaM, indicating that inhibition of the mutants by CaV1p1 may be both competitive and noncompetitive, as seen in the wild-type enzyme.

In the absence of Ca\(^{2+}\)/CaM, the \(\Delta40\) and \(\DeltaC33\) mutants had significant NO formation activities, whereas wild-type nNOS was active only in the presence of Ca\(^{2+}\)/CaM as described previously (8, 9). In the absence of CaM, addition of CaV1p1 to the mutant systems caused only slight inhibition (Table II).

### Table II

| CaV1p1 peptide concentration (\(\mu M\)) | NO formation activity | NADPH oxidation activity |
|----------------------------------------|----------------------|--------------------------|
| – CaV | + CaV | IC\(_{50}\) \(\mu M\) | – CaV | + CaV |
| Wild-type | | | | |
| – CaM | 14.1 \pm 0.9 | 3.4 \pm 0.4 (24%) | 1.8 | 65 \pm 8 | 15 \pm 3 (24%) |
| + CaM | | | | |
| \(\Delta40\) | 2.1 \pm 0.2 | 1.8 \pm 0.1 (86%) | >20 | 18 \pm 1 | 8 \pm 2 (45%) |
| \(\DeltaC33\) | 10.6 \pm 1.2 | 3.8 \pm 0.2 (38%) | 7.0 | 44 \pm 2 | 18 \pm 2 (41%) |
| – CaM | 2.5 \pm 0.1 | 2.2 \pm 0.1 (88%) | >20 | 44 \pm 1 | 15 \pm 2 (35%) |
| + CaM | 8.5 \pm 0.5 | 5.5 \pm 0.2 (65%) | 4.5 | 64 \pm 7 | 40 \pm 4 (63%) |

\(a\) Addition of 5 \(\mu M\) CaV1p1 peptide.

\(b\) The concentration of CaV1p1 peptide to show 50% inhibition of activity.

\(c\) Not detectable.

\(d\) The parentheses contain the percent residual activity in the presence of CaV1p1 relative to that in the absence of CaV1p1.
mutants, the NADPH oxidation rates did not correlate with the NO formation activities in the absence of CaM, which was consistent with previous reports (17, 33).

**DISCUSSION**

Within the NOS dimer, the electrons required for NO formation are transferred from NADPH to FAD, and from FAD to FMN in the reductase domain of the same subunit, and are then finally transferred from FMN to the heme in the oxygenase domain of the other subunit (39, 40). Ca²⁺/CaM binding activates both inter- and intradomain NOS electron transfers through conformational rearrangements between the oxygenase and reductase domains and within the reductase domain itself. In the present study, we have investigated the multiple inhibitory mechanisms of caveolin action on electron transfer in nNOS using the wild-type enzyme and various mutants. We expressed rat caveolin-1 and caveolin-3 proteins in E. coli and examined the effects of these recombinant proteins on NO formation by nNOS. Full-length caveolin-1 inhibited the activity, but not to the degree previously reported for caveolin peptides (30), probably due to the formation of aggregates in solution. Full-length caveolin-3 also did not show significant inhibition of the NO formation activity probably because of its aggregation (data not shown). However, both caveolin-1 and caveolin-3 directly interacted with nNOS in *in vitro* binding assays (Fig. 3, A and B). In mammalian cells, both caveolins are localized with eNOS and nNOS in caveolae and also interact with other proteins that might influence the effect of caveolins on NOS activities.

The caveolin-1 scaffolding domain peptide, CaV1p1 (residues 82–101) efficiently inhibited NO formation and NADPH oxidation by wild-type nNOS in the presence of Ca²⁺/CaM. These results were consistent with previous reports on eNOS (19). This nNOS inhibition was not completely reversed by addition of excess Ca²⁺/CaM (Fig. 5B), and double reciprocal plots of the data revealed that CaV1p1 appears to be a mixed-type inhibitor, acting as both a competitive and noncompetitive inhibitor for CaM-dependent nNOS activation (Fig. 5C). In contrast, previous reports have indicated that the inhibition of eNOS activity by the same peptide was competitive and completely reversed by Ca²⁺/CaM (24). These results suggest that the inhibition mode of nNOS by caveolin is different from that of eNOS and that CaV1p1 does not directly interact with CaM and the CaM binding site (discussed below).

Our *in vitro* binding assay results indicate that both the oxygenase and reductase domains of nNOS independently interact with full-length caveolin-1 and caveolin-3 proteins, and that the CaM binding site and CaM itself are not essential for this interaction (Fig. 3, A and B, respectively). We further characterized the binding site of caveolin-1 in the reductase domain of nNOS by examining reductase domain truncation mutants (Fig. 7). A fragment of the reductase domain containing only the FMN domain could bind caveolin-1, suggesting that the FMN domain itself is essential for the caveolin-1 interaction. As shown in Fig. 7, then NOS reductase domain in which the autoinhibitory domain was deleted (RedΔ40) also interacted with caveolin-1, suggesting that these 40 amino acids located in the FMN domain are not required for the interaction between caveolin-1 and the nNOS reductase domain.

Competitive binding experiments with CaV1p1 revealed (Fig. 3C) that the scaffolding domain-containing sequences (CaV1p1) in caveolin-1 form a domain that interacts with both the oxygenase and reductase domains of nNOS. Addition of 5–10-fold excess CaM to the binding mixture did not eliminate the interaction (Fig. 4, A and B), suggesting that CaM does not compete well with caveolin for binding to nNOS, unlike for binding to eNOS. The proteins interacting with caveolin have been known to contain a consensus binding motif that is also present in the NOS oxygenase domain. It was reported that site-directed mutagenesis of the binding motif within eNOS totally blocked the ability of caveolin-1 to interact with eNOS and suppressed NO release (29, 30). In contrast, our F584L and W587L mutations in the corresponding nNOS motif resulted in only a partial loss of the CaV1p1 inhibitory effect (Table 1). These results indicate that the caveolin-1 binding motif affects the interaction with CaV1p1, but is not essential for binding of caveolin-1 to nNOS. The crystal structures of the eNOS and nNOS oxygenase domains show that the caveolin-1 binding motif is located in the heme distal site and is almost buried within the protein (6), implying that this motif is unlikely to interact directly with caveolin-1.

CaV1p1 completely inhibited the cytochrome c reductase activity of the isolated reductase domain regardless of the presence or absence of the CaM binding site (Fig. 6A). These results indicate that CaV1p1 inhibits intradomain electron transfer within the nNOS reductase domain independent...
from the oxygenase domain, and that the CaM binding site is not essential for this inhibition. It is noteworthy that CaV1p1 inhibited 50% of the nNOS ferricyanide reductase activity, which is in direct contrast to the case of eNOS, which did not show a change in ferricyanide reductase activity under similar conditions (31). Because cytochrome c accepts electrons from FMN, whereas ferricyanide can accept from both FAD and FMN, these data suggest that electron transfer from FAD to FMN and/or from FMN to both exogenous electron acceptors is inhibited by the binding of CaV1p1 to nNOS (Fig. 9). In a manner similar to that seen in the case of inhibition of NO formation, the inhibition of cytochrome c reduction was not completely reversed by addition of excess Ca2+/CaM, whereas the inhibition of ferricyanide reduction was recovered by addition of excess Ca2+/CaM (Fig. 6C). In the case of eNOS, the inhibition of cytochrome c reductase activity was fully reversed by excess Ca2+/CaM (31). Taken together, these data indicate that the interaction of nNOS with CaV1p1 and the mechanism of inhibition have some features in common with eNOS, but that several important differences exist.

Several isoform-specific structural elements have been found to control the Ca2+/CaM-dependent activation of nNOS, including the autoinhibitory loop in the FMN subdomain, the C-terminal extension, and various regulatory phosphorylations. In the absence of Ca2+/CaM, the autoinhibitory and C-terminal control elements repress electron transfers within and from the reductase domain in both nNOS and eNOS (11-17). The nNOS control element deletion mutants (Δ40 and ΔC33) retained activity even in the absence of Ca2+/CaM (16, 17) (Table II). In this study, the effects of the caveolin peptide on these CaM-independent electron transfers were investigated using Δ40 and ΔC33. The addition of CaV1p1 had only a slight effect on the CaM-independent NO formation activities of these mutants, whereas CaM-dependent NO formation was mostly inhibited, similar to the effect on the wild-type enzyme (Table II). These results suggest that CaV1p1 interacts with (a) site(s) other than these two control elements and can affect the CaM-induced conformational changes that activate NO synthesis in the mutant enzymes. On the other hand, the reductase activities of these mutants were inhibited by CaV1p1 both in the presence and absence of CaM (data not shown), suggesting that caveolin affects intradomain electron transfer within the reductase domain independent of CaM, thus differing from the case of interdomain electron transfer. It should be stressed that inhibition of the catalytic activities of the wild-type enzyme and the Δ40 and ΔC33 mutants of nNOS can be partially reversed by addition of excess Ca2+/CaM in a mixed-inhibition manner. This suggests that CaV1p1 interacts with both CaM-bound and CaM-free nNOS at multiple sites, and that it directly and indirectly affects CaM-dependent activation. The interactions between nNOS and caveolin might also be affected by other protein-protein interactions. Gratton et al. (41) indicated that the conformational change that occurred in eNOS following the addition of CaM is not sufficient to disrupt its physical association with caveolin-1, and that the presence of hsp90 facilitates the actions of CaM in displacing eNOS from caveolin-1. Hsp90 has been shown to activate nNOS by enhancing Ca2+/CaM binding in vitro and in vivo (42), suggesting that it may also affect caveolin-dependent inhibition. It has been reported that phosphorylations in eNOS and nNOS affect electron transfers, catalytic activities, and CaM binding (43–45). In the cell, many other proteins interact with NOS and regulate its activity (46). Therefore, in order to totally understand the regulation mechanism of NOS by caveolin, we will need to investigate further to obtain more information on this subject area.

In summary, we demonstrated that: (i) the scaffolding domain of caveolin-1 independently interacts with both the oxygenase and reductase domains of nNOS; (ii) the scaffolding domain peptide of caveolin-1 (CaV1p1; residues 82–101) inhibited electron transfers mainly from FAD to FMN in the reductase domain and from FMN to heme between the reductase and oxygenase domain; (iii) excess CaM reversed the inhibition only partially, suggesting that caveolin-1 conferred a mixed-type inhibition (both competitive and noncompetitive) to CaM-induced nNOS activity; and (iv) CaV1p1 inhibited CaM-dependent NO formation in full-length nNOS but did not affect CaM-independent activities.

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