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Inhibition of Neuronal Nitric Oxide Synthase by Phosphatidylinositol 4,5-Bisphosphate and Phosphatidic Acid[1]

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Phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidic acid (PA) were found to inhibit strongly the citrulline formation activity of neuronal nitric oxide synthase (nNOS; EC 1.14.13.39). Such inhibition was not observed with any other phospholipid examined. A kinetic analysis of purified nNOS showed no significant change in apparent K_m for L-Arg or NADPH caused by these inhibitory phospholipids. Electron paramagnetic resonance analysis revealed no significant spectral perturbation of the ferriheme or flavin semiquinone upon the addition of PIP2. On the other hand, a lower enhancement of the NADPH diaphorase activity by Ca^{2+}-calmodulin was observed in the presence of PIP2 and PA, and the citrulline formation activity was protected from phospholipid inhibition by preincubation with Ca^{2+}-calmodulin. Moreover, trypsin digestion analysis showed that the cleavage site within the calmodulin-binding site of nNOS was specifically protected from trypsin by the addition of PIP2 and PA. These results strongly suggest that PIP2 and PA inhibit the citrulline formation activity of nNOS by blocking the interaction of the enzyme with Ca^{2+}-calmodulin.

Key words: calmodulin, nitric oxide, nitric oxide synthase, phospholipid.

Nitric oxide (NO) has diverse physiological functions; for example, it acts as a signaling molecule in the nervous and cardiovascular systems, and an antimicrobial agent in the immune system (*1-7*). It is generated from L-arginine by a family of enzymes called nitric oxide synthases (NOS, EC 1.14.13.39) (*8-10*). Two general categories of NOS are known: constitutive enzymes that are regulated by Ca^{2+}-calmodulin (*11*), and Ca^{2+}-independent inducible enzymes with a tightly bound calmodulin (*12*). Both types of NOS contain a heme binding domain and a cytochrome P450 reductase-like domain connected by a polypeptide that serves as the calmodulin binding site (*13*). The P450 reductase domain contains FMN and FAD as cofactors involved in electron transfer from NADPH to the heme center (*14*), where NO is produced from the substrates L-Arg and molecular oxygen (*15-20*). Calmodulin binding to NOS facilitates electron transfer from NADPH to the heme site, thereby activating NO production, and also increases the NADPH-dependent reduction rate of artificial electron acceptors (*21*). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin (H4B) is also required as a cofactor for full enzymatic activity (*22*), and more recent structural studies of the heme binding domain of inducible NOS have shown that H4B is bound in close proximity to the heme site in the vicinity of the dimer interface (*19, 20*).

Neuronal NOS (nNOS) is a constitutive enzyme that is fully dependent on Ca^{2+}-calmodulin. Purification (*11, 23-25*), cloning (*13, 26*), and expression (*17, 27-32*) of native and recombinant nNOS have been reported. Neuronal NOS has a unique N-terminal extension with the PDZ motif (*33, 34*), in contrast to other NOS isoforms. Mammalian nNOS has been implicated in the regulation of neuronal cell biology and neurotransmission (*35, 36*) as a major nonadrenergic-noncholinergic transmitter in enteric nerves (*37, 38*), in the neuroendocrine biology of the hypothalamus and pituitary (*39*), in modifying skeletal muscle contractile force and development (*33, 40, 41*), and in controlling total body sodium content and body fluid homeostasis *via* its expression in the macula densa and distal nephron of the kidney (*42*).

Although nNOS has usually been purified from cytosolic fractions (*11*), it has also been obtained from the precipitate fraction of cerebellum homogenate (*43*). The properties of the enzyme purified from the precipitate seem to be the same as those of the enzyme from the cytosolic fraction (*44*). The PDZ domain of nNOS has been reported to interact with postsynaptic density proteins (PSD-95 and PSD-93) (*34*) and PIN (*45*). Furthermore, a recent im-

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; H_4B, (6*R*)-5,6,7,8-tetrahydrobiopterin; PIP2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; SM, sphingomyelin; DCPIP, dichlorophenol-indophenol.

munoelectron and confocal microscopical study showed that nNOS is mainly localized in synaptic vesicles, with a dense accumulation associated with the plasma membrane of dendrites (*46*). Thus, the nNOS protein seems to interact with the biological membrane in the nervous system. The reported differences in the behavior of the enzyme during purification may thus be due to its association with phospholipids, as well as to protein-protein interactions. We have previously reported that the existence of phospholipid(s) in the supernatant of rat cerebellum homogenate strongly affects the citrulline formation activity of nNOS in the supernatant (*47*).

In this paper, we show that phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidic acid (PA) tightly bind to nNOS and inhibit its activity by blocking the binding of Ca^{2+}-calmodulin to the enzyme.

EXPERIMENTAL PROCEDURES

Materials—Calmodulin, FAD, FMN, L-arginine, L-citrulline, phospholipids [PIP2, PA, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylserine (PS), and sphingomyelin (SM)], and calmodulin agarose were from Sigma, and 2′,5′-ADP Sepharose 4B from Pharmacia Biotech. H4B was obtained from the Schircks Laboratory (Jona, Switzerland), and [^{14}C-*U*]-L-arginine (11.84 GBq/mmol) from Dupont, New England Nuclear. Water was purified with a Milli-Q purification system (Millipore). Other chemicals used in this study were of analytical grade.

Esherichia coli Expression System of Mouse nNOS and Purification of Recombinant nNOS—Recombinant mouse nNOS was obtained from an *E. coli* expression system as reported previously (*29, 30*). Purification was conducted as reported elsewhere with slight modifications (*48*); Arg and H4B were present at concentrations of 200 and 30 μM, respectively, throughout the purification. The purified enzyme was stored at −80°C in buffer composed of 50 mM Tris-HCl (pH 7.4), 100 μM DTT, 1 mM EGTA, 0.1 mM EDTA, 10 μM Arg, 10 μM H4B, and 10% glycerol. The amount of purified nNOS protein was estimated by the Coomassie Protein Assay Reagent (Pierce Chemical) with bovine serum albumin as a standard. Since glycerol strongly influences the inhibition of the citrulline formation activity by phospholipids, nNOS stored in buffer containing 10% glycerol was passed through a Sephadex G-25 column to remove glycerol before use.

Preparation of Phospholipids—Phospholipids were dissolved in chloroform, and completely dried *in vacuo*. The pellet was then suspended in 50 mM Tris-HCl (pH 7.4) to a final concentration of 10 mg/ml, and then briefly sonicated on ice for 2 min. This suspension was used immediately in experiments.

Phospholipid Vesicles Absorption Test—Purified nNOS (100 μg) and phospholipid vesicles (20 or 400 μg) were mixed in a total volume of 100 μl buffer [50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 100 μM DTT, 10 μM Arg, 10 μM H4B, 5 μM FAD, and 5 μM FMN], incubated on ice for 30 min, and then centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant fraction was mixed with 100 μl of SDS-loading buffer, and a 10 μl aliquot was analyzed by 7.5% SDS-polyacrylamide gel electrophoresis (*49*). The precipitate was washed with 200 μl of the same buffer three times to remove non-specifically bound enzyme. The resultant precipitate was dissolved in 200 μl of SDS-loading buffer, and a 10 μl aliquot was analyzed by 7.5% SDS-polyacrylamide gel electrophoresis (*49*).

Assay of Citrulline Formation by nNOS—The formation of [^{14}C-*U*]-L-citrulline from [^{14}C-*U*]-L-arginine by nNOS was measured by thin-layer chromatography according to the method reported previously (*47*), as follows: The standard assay was performed in an assay mixture containing 16.7 mM HEPES-NaOH (pH 7.4), 4.2 mM Tris-HCl (pH 7.4), 667 μM EDTA, 167 μM EGTA, 0.1 mM DTT, 16.7 μM [^{14}C-*U*]-L-Arg, 667 μM NADPH, 1.2 mM $CaCl_2$, 100 ng of calmodulin, 1.25 μM FAD, 1.25 μM FMN, 2.5 μM H4B, and appropriate amounts of nNOS and phospholipid, in a total volume of 30 μl. The reaction mixture was incubated for 5 min at 25°C, and then 10 μl each of 10 mM cold L-Arg and 10 mM L-citrulline were added as standard markers. The mixtures were boiled for 90 s, after which 5 μl aliquots were spotted onto a Chromagram cellulose plate (Eastman Kodak) and separated by thin layer chromatography developed with methanol-pyridine-H_2O (20:1:5, v/v/v). L-Arg and L-citrulline were detected by the ninhydrin reaction, and radioactivity was measured by autoradiography with a Fuji BAS2000 Bioimaging analyzer.

Pre-incubation with Ca^{2+}-calmodulin was done as follows: 17 μg of nNOS and 51 μg of Ca^{2+}-calmodulin were incubated on ice for 15 min in 100 μl of buffer [50 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$, 100 μM DTT, 10 μM Arg, 10 μM H4B, 5 μM FAD, and 5 μM FMN]. This mixture was used to assay citrulline formation.

Electron Paramagnetic Resonance (EPR) Measurements—Absorption spectra were recorded on a Hitachi U3210 spectrophotometer or a Beckman DU-7400 spectrophotometer. Electron paramagnetic resonance (EPR) measurements were carried out on a JEOL JEX-RE1X spectrometer equipped with an Air Products model LTR-3 Heli-Tran cryostat system, with temperature monitored by a Scientific Instruments series 5500 temperature indicator/controller as reported previously (*50*). All spectral data were processed by a computer using KaleidaGraph software ver. 3.05 (Abelbeck Software).

Assay of Protection against Limited Proteolysis by Trypsin—Limited proteolysis by trypsin was carried out according to the reported method (*51*) in the presence or absence of phospholipid; purified nNOS and phospholipid were preincubated on ice for 15 min, and then trypsin was added.

NADPH-DCPIP Oxidoreductase Activity—P450 reductase activity was measured in terms of the activity of NADPH-dependent DCPIP reduction. The standard assay buffer contained 50 mM Tris-HCl (pH 7.4), 100 μM DTT, 1 mM $CaCl_2$, 5 μM FAD, 5 μM FMN, 10 μM H4B, 35 μM DCPIP, and an appropriate amount (0-30 μg/ml final concentration) of phospholipid. The assay was performed by the sequential addition of purified nNOS, NADPH, and calmodulin. The final concentrations of nNOS and NADPH were 0.44 μg/ml and 100 μM, respectively. The rate of DCPIP reduction was monitored by measuring the absorbance change at 600 nm at 25°C with a Hitachi U3210 spectrophotometer equipped with a thermostatted cell holder.

RESULTS

Phospholipid Vesicle Absorption Test and the Inhibition of the Citrulline Formation Activity by Phospholipid—To investigate whether various phospholipids bind to nNOS, a phospholipid vesicle absorption test was carried out with commercially obtained authentic phospholipids. Figure 1 shows the results of SDS-polyacrylamide gel electrophoresis. In the control, without phospholipids, the enzyme was recovered in the supernatant fraction after 100,000 × *g* centrifugation. Upon the addition of vesicles composed of PIP2, PA, or PS, purified nNOS was recovered in the precipitate, and remained tightly bound to the vesicles after three washes with buffer. On the other hand, nNOS was not absorbed by other phospholipids such as PC, PI, or SM, and remained in the supernatant fraction. The enzyme was partially absorbed by vesicles composed of PE or PG, although the major part of the precipitated enzyme was removed from the vesicles by the washing procedure. A phospholipid vesicle absorption test with PIP2, PA, or PS was also carried out at lower phospholipid concentrations (Fig. 1C). Since vesicles composed of PIP2 are very brittle, part of the absorbed enzyme was washed out. However, the main part of the enzyme was clearly absorbed by PIP2, and in the cases of PA or PS, nNOS was completely absorbed at the tested concentrations. These results show that nNOS has a selective affinity for PIP2, PA, and PS.

To study the effect of phospholipid on nNOS, the citrulline formation activity was measured in the presence of various phospholipids at 0-500 μg/ml concentrations (Fig. 2, A, B and C). Before the experiments, the nNOS was passed through a Sephadex G-25 gel-filtration column to remove glycerol. When PIP2 was added to the reaction mixture, the citrulline formation activity of nNOS was dramatically inhibited in a dose-dependent manner, and similar inhibition was also observed with PA, although the dose-dependence was different (Fig. 2, A, B, and C). In contrast, other phospholipids (PC, PE, PG, PI, and SM) did not inhibit nNOS at any concentration tested, except PS, which was slightly inhibitory at high concentration (>100 μg/ml) (Fig. 2B). As described above, nNOS was partially absorbed by vesicles composed of PE or PG, but not inhibited by these phospholipids. This result suggests that the interaction between nNOS and phospholipid may be reversible in the presence of calmodulin.

Effect of PIP2 and PA on the Heme-Binding Domain—To clarify the inhibition mechanism, kinetic studies of the citrulline formation activity were carried out in the presence or absence of phospholipids. The obtained parameters, K_m for Arg and V_{max}, are summarized in Table I. The apparent K_m for Arg did not change, whereas the V_{max} decreased with an increase in phospholipid concentration.

The effect of inhibitory phospholipids on the ferriheme center of purified nNOS was further investigated by EPR spectroscopy, which is a sensitive technique that detects small changes at the ferriheme site of NOS isoforms (*52, 53*) (Fig. 3). The EPR spectrum at 10 K of the ferriheme center of the resting enzyme showed signals of a predominant high-spin species at $g_{1,2,3}=7.61$, 4.08, and 1.81, in addition to a sharp flavin semiquinone radical signal at $g=2.0$ and a very small signal of low-spin ferriheme species (Fig. 3A). The rhombicity (defined in terms of the ratio of the rhombic and axial zero field splitting parameters, E/D) of the predominant high-spin ferriheme species is consistent with the EPR properties of the pentacoordinated ferriheme center in L-Arg- and H_4B-binding mouse nNOS, in which L-Arg is bound without direct coordination to the ferriheme (*52, 53*). Upon the addition of excess substrate, a small shift in the EPR signal at $g_1=7.61$ to 7.59 was observed ($E/D=0.073$), probably due to the presence of a small amount of substrate-free high-spin ferriheme species in the as-isolated enzyme that is not resolved in the EPR spectrum (data not shown).

The addition of an excess amount of PIP2 (300 μg/ml) to the resting enzyme, causing complete inhibition of its

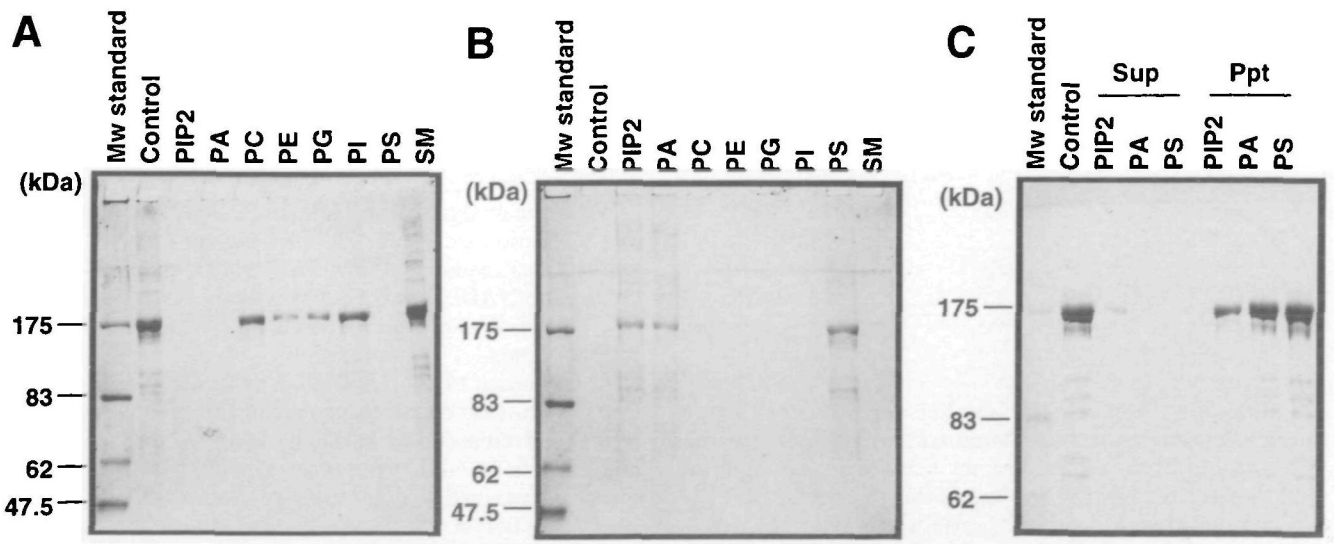

Fig. 1. **Phospholipid vesicle absorption test.** The affinity of purified nNOS for various kinds of phospholipid vesicles was tested as described in "EXPERIMENTAL PROCEDURES." The supernatant and precipitate fractions were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining. Panel A, supernatant fraction; panel B, precipitate fraction after washing three times with buffer. Panel C, phospholipid vesicle absorption test with a lower concentration (200 μg/ml) of phospholipids.

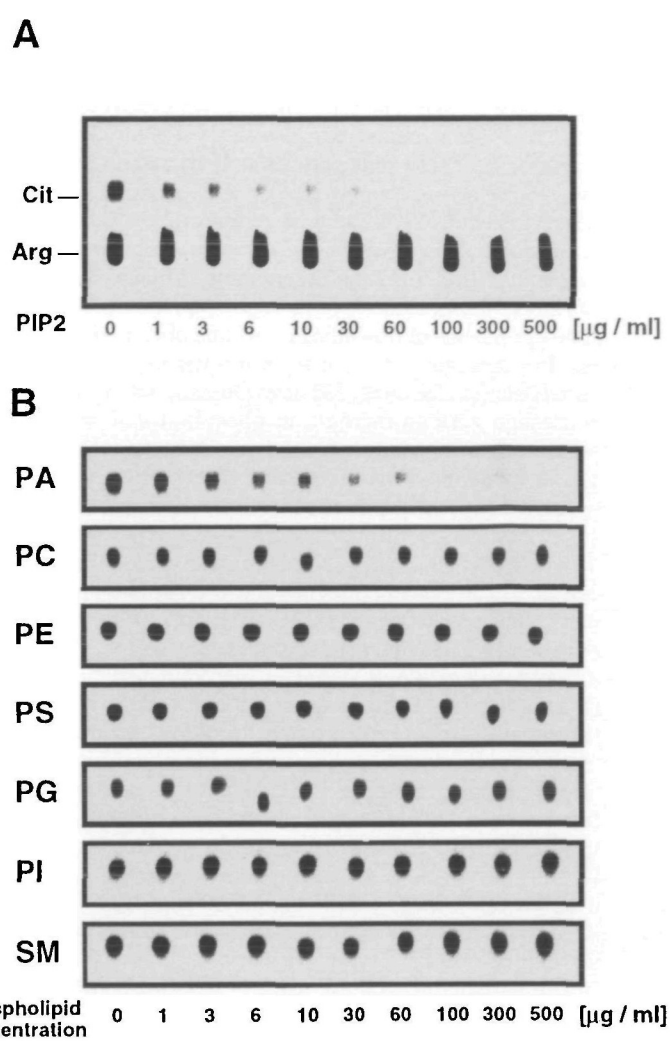

Fig. 2. **Effect of various phospholipids on the citrulline formation activity of mouse nNOS.** Panel A: Inhibition of the citrulline formation activity of mouse nNOS by PIP2. As described in "EXPERIMENTAL PROCEDURES," the citrulline formation reaction was carried out in the presence or absence of various concentrations of PIP2. ^{14}C-Amino acids (L-Cit and substrate L-Arg) were separated by thin layer chromatography, and analyzed by autoradiography. Panel B: Effect of various phospholipids on the citrulline formation activity. The citrulline formation reactions were carried out in the presence or absence of various phospholipids. This figure shows the autoradiogram of ^{14}C-Cit formed. The concentrations of each phospholipid are shown at the bottom of the panel. Panel C: Dose dependencies of the inhibition of citrulline formation by PIP2 and PA. ^{14}C-Cit formed was quantified with a BAS 2000 image analyzer. Various concentrations of PIP2 and PA not shown in panel A and B were also analyzed for this figure. Inset: Regions for 0-20 μg/ml PIP2 and PA are replotted. These data are the results for one of three independent experiments.

TABLE I. **Kinetic parameters for Arg.** Kinetic parameters for Arg in the citrulline formation reaction were measured in the presence or absence of PIP2 or PA. [a]"none" is the control without phospholipids.

	K_m (μM)	V_{max} (nmol/mg/min)
none[a]	1.2	330
+PIP2		
1 μg/ml	1.1	158
10 μg/ml	1.2	71
+PA		
3 μg/ml	1.1	167
30 μg/ml	1.1	85

citrulline formation activity (see Fig. 2A), did not change the EPR lineshapes of the predominant high-spin ferriheme or the stable flavin radical species (Fig. 3B), as compared with those of the resting enzyme in the absence of PIP2. It is concluded that excess PIP2 does not modify the ferriheme center of the purified enzyme, which is in line with the kinetic finding of no change in the K_m for L-Arg in the presence or absence of PIP2 (Table I).

Effects of PIP2 and PA on the Sensitivity of the P450 Reductase Domain of nNOS to Ca^{2+}-Calmodulin—The reducing equivalents for citrulline formation by nNOS are supplied by NADPH at the cytochrome P450 reductase domain, which also shows NADPH-dependent diaphorase activity with several artificial electron acceptors including dichlorophenol-indophenol (DCPIP), cytochrome *c*, and ferricyanide (*21*).

A kinetic analysis of the effect of NADPH on the citrulline formation activity in the presence or absence of PIP2 and PA was made. As shown in Table II, the apparent K_m values for NADPH did not change whereas the apparent V_{max} values became smaller in the presence of inhibitory phospholipids.

The kinetics of the NADPH-diaphorase activity of the P-450 reductase domain were also examined using DCPIP as an electron acceptor in the presence or absence of PIP2 and PA. We found that the activation of nNOS by Ca^{2+}-calmodulin was reduced in the presence of PIP2 and PA (described below, see Fig. 4), although the apparent K_ms for NADPH did not change significantly (Table II). In the absence of inhibitory phospholipid, the electron transfer from NADPH to DCPIP through the P450 reductase domain was activated approximately 3.3-fold by the addi-

tion of calmodulin (Fig. 4), a value consistent with previous findings (*18, 21, 54*). In the presence of PIP2, the activation of the NADPH-diaphorase activity by Ca^{2+}-calmodulin decreased gradually with increasing phospholipid concentration (Fig. 4A). At a concentration of 10 μg/ml PIP2, the activation by calmodulin was almost lost, and the basal activity in the absence of calmodulin was partially inhibited (approximately 65% of the activity in terms of the initial velocity, see Table II). This inhibition mechanism is less clear, because the electron-transfer site(s) of nNOS for DCPIP are not known. Similar results were obtained with PA (Fig. 4B). These results suggest that both PIP2 and PA inhibit the binding of nNOS with Ca^{2+}-calmodulin.

In order to test if this is the sole reason for the inhibition of the citrulline formation activity of nNOS by inhibitory phospholipids, the effect of pre-incubation of the purified enzyme with Ca^{2+}-calmodulin on the inhibition of the citrulline formation activity by PIP2 and PA was investigated. Figure 5 shows that pre-incubation of the enzyme with Ca^{2+}-calmodulin decreased the extent of the inhibition of citrulline formation activity by PIP2 and PA. At higher

TABLE II. **Kinetic parameters for NADPH.** Kinetic parameters for NADPH in the citrulline formation reaction and NADPH-diaphorase reaction were measured in the presnce or absence of phosphlipids. [a]"none" is the control without phospholipids.

Citrulline formation

	K_m (μM)	V_{max} (nmol/mg/min)
none[a]	0.8	510
+PIP2 (1 μg/ml)	0.8	170
+PA (3 μg/ml)	0.8	230

NADPH-diaphorase

	With calmodulin		Without calmodulin	
	K_m (μM)	V_{max} (μmol/mg/min)	K_m (μM)	V_{max} (μmol/mg/min)
none[a]	0.7	19.5	0.7	6.1
+PIP2 (1 μg/ml)	0.7	4.3	0.7	4.0
+PA (3 μg/ml)	0.7	4.5	0.8	4.1

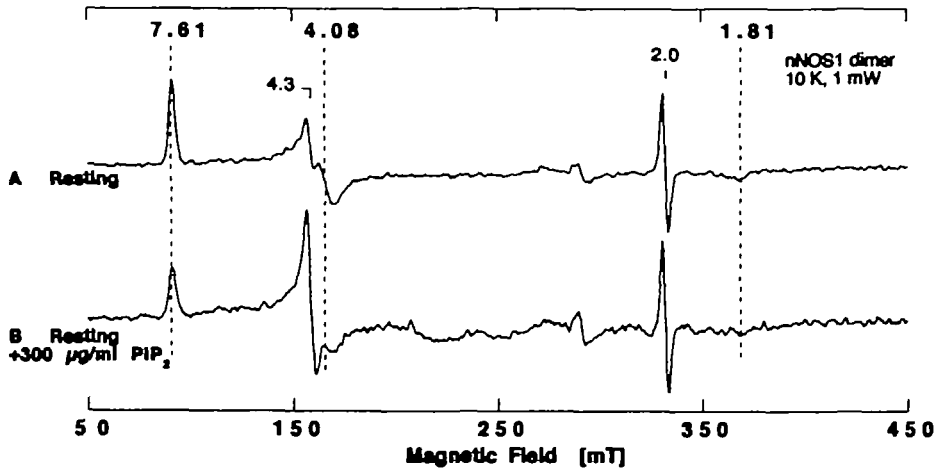

Fig. 3. **EPR spectra at 10 K of purified nNOS in the absence (trace A) and presence (trace B) of excess PIP2.** The resting enzyme was purified in the presence of H_4B and L-Arg as described in "EXPERIMENTAL PROCEDURES." The EPR spectrum at 10 K of the resting enzyme (as isolated; trace A) was compared with that of the PIP2-inhibited enzyme in the presence of excess PIP2 (300 μg/ml; trace B). Instrument settings: microwave power, 1 mW; modulation amplitude, 1 mT; the g values are indicated in the figure.

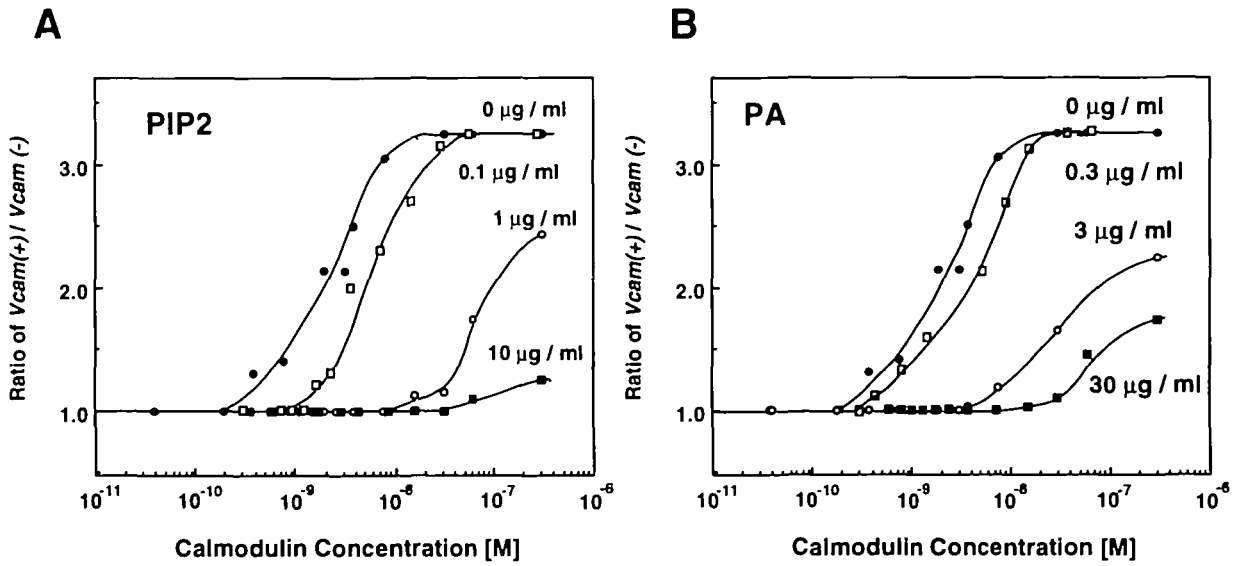

Fig. 4. **PIP2 and PA inhibit the activation of NADPH-diaphorase activity by Ca^{2+}-calmodulin.** The activation of NADPH-diaphorase activity ($Vcam(+)/Vcam(-)$) was measured with various concentrations of calmodulin and phospholipids. This figure shows the average of four independent experiment. Symbols show the concentrations of phospholipids;, ●, 0 μg/ml; □, 0.1 μg/ml; ○, □, 1 μg/ml, and ■ 10 μg/ml. Panel A, PIP2; panel B, PA.

concentrations of inhibitory phospholipids, however, the protective effect of Ca^{2+}-calmodulin was lowered, and it was completely abolished at concentrations higher than 10 μg/ml (Fig. 5).

It should be noted that the dose dependencies of the inhibition of the citrulline formation activity by PIP2 and PA (see Fig. 2) coincide with those of the NADPH-dependent diaphorase activity (see Fig. 4); the concentration of calmodulin in the inhibition experiments on citrulline formation shown in Fig. 2 was 210 nM. It is therefore concluded that the strong inhibition of nNOS activity by PIP2 and PA is mainly caused by inhibition of the binding of Ca^{2+}-calmodulin to the calmodulin-binding site.

Effect of Phospholipids on the Limited Proteolysis of nNOS by Trypsin—It has been reported that rat nNOS is cleaved at the calmodulin-binding site upon partial digestion with trypsin, affording two domains, the heme binding domain (p90 fragment) and the P450 reductase domain (p80 fragment) (*51, 55, 56*). In light of these reports, the effect of phospholipids on limited proteolysis by trypsin was tested using purified mouse nNOS (Fig. 6), which has a high degree of amino acid sequence identity with the rat enzyme. The digestion pattern of mouse nNOS by trypsin in the absence of phospholipid was identical to that of the rat enzyme reported by others (*51, 55, 56*); a band equivalent to the p80 fragment, which corresponds to the P450 reductase domain, was observed after limited proteolysis and was considered to have been generated by cleavage at the calmodulin-binding site. Upon the addition of PIP2 or PA, the relative intensity of the p80 fragment was clearly reduced. On the other hand, this phenomenon was not observed with PC. These results suggest that the cleavage site within the calmodulin-binding site of nNOS is specifically protected from trypsin by the addition of PIP2 and PA, a finding consistent with the results of kinetic analysis described above.

The trypsin digestion analysis also revealed a decrease in the amount of the p90 fragment and an increase in the amount of the p135 fragment. This seems to indicate that PIP2 and PA at the tested concentrations interact not only with the calmodulin-binding site, but also with other regions, such as the connection region linking the N-terminal PDZ domain and the rest of the heme-binding domain. An increase in p135 fragment generation was also observed upon the addition of PC. Unfortunately, the structure of the connection region linking the N-terminal PDZ domain and the heme-binding domain has not been reported. Thus, it is unkown which amino acid residues are located on the molecular surface of the enzyme. Therefore, the mechanism for the increase in the amount of p135 fragment is not clear.

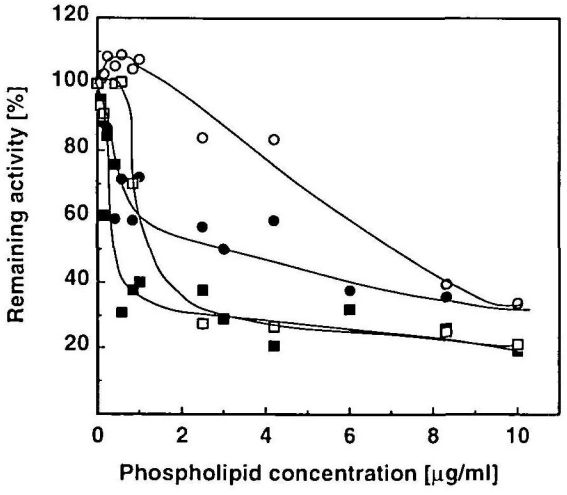

Fig. 5. **Effect of pre-incubation with Ca^{2+}-calmodulin on the inhibition by phospholipid.** Purified nNOS was pre-incubated with Ca^{2+}-calmodulin, and the citrulline formation activity was measured in the presence of various concentrations of PIP2 (□) and PA (○). The controls without pre-incubation are also shown; ■, PIP2 without pre-incubation, ●, PA without pre-incubation. This figure shows the results of one of three independent experiments.

DISCUSSION

In this paper, we demonstrate that PIP2 and PA strongly bind to the calmodulin binding site of nNOS, and inhibit the citrulline formation activity. This inhibition mechanism is supported by the results of the DCPIP assay of the P450 reductase domain, the effect of pre-incubation with calmodulin on citrulline formation, and the protection against limited proteolysis by trypsin afforded by these phospholipids. Further, the apparent K_m values for Arg and NADPH, and the EPR spectrum of the ferriheme and the stable flavin radical species remained unchanged in the

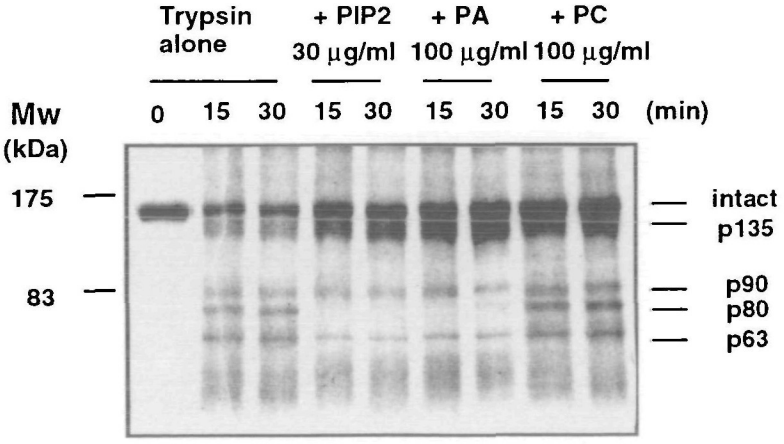

Fig. 6. **Effect of phospholipids on limited proteolysis by trypsin.** Limited proteolysis of mouse NOS by trypsin was carried out in the absence or presence of phospholipids according to Lowe *et al.* (*51*). The proteolysis was started by the addition of trypsin, and stopped after 15 or 30 min; the products were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis.

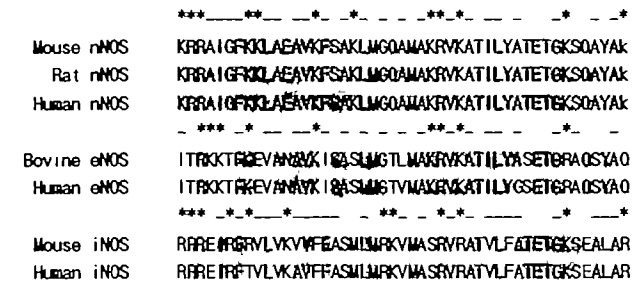

Fig. 7. **Amino acid sequence alignments of the calmodulin binding sites of NOS isoforms.** The amino acid sequence of the calmodulin binding site of mouse NOS is compared with those of other NOSs. Identical amino acid residues are shadowed, and amino acid residues conserved between isoforms are marked as * (basic amino acids) or - (hydrophobic amino acids). This figure is based on data by Bredt *et al.* (*9, 13*).

presence of inhibitory phospholipids.

This inhibition can be attributed to the negative charge(s) on the phospholipids and the hydrophobic interaction between nNOS and phospholipid, as judged from the dose dependency of the inhibition of citrulline formation by phospholipids; PIP2 has two phosphate groups, and strongly inhibits the citrulline formation activity of nNOS at a lower concentration than do PA and PS, which have only one negative charge. Several proteins are specifically bound to phosphatidylinositol phosphates, such as pleckstrin homology domain-containing proteins (*57, 58*) and calcium-dependent activator protein for secretion (*59*). In comparison with these proteins, the phospholipid binding specificity of nNOS is relatively broad, consistent with the idea that the interaction with phospholipids is due to negative charge(s).

Figure 7 shows amino acid sequence alignments of the calmodulin binding sites of NOS isoforms. The consensus motif in the calmodulin binding site is composed primarily of several hydrophobic and basic amino acid residues. These residues are probably involved in the interaction with acidic phospholipids. This is in line with the recent report by Matsubara *et al.*, who showed that a synthetic peptide corresponding to the calmodulin binding site of nNOS is absorbed on vesicles of PS, but not PC (*60*). Inhibition by acidic phospholipids through interaction with the calmodulin-binding site has also been reported for eNOS, which is the membrane-bound constitutive NOS isoform (*61*). The high degree of amino acid sequence homology between nNOS and eNOS suggests that the inhibition mechanism described here may also be applicable to eNOS (*61-70*), although the interaction of the latter enzyme with phospholipids has not been investigated in detail. Since interactions between the calmodulin binding site and phospholipid have been reported not only for NOS but also for several other calmodulin-dependent proteins, such as calponin (*71*), Ca^{2+}-ATPase (*72*), and human growth-associated protein-43 (*73*), interaction with phospholipids is likely to be applicable in the case of other calmodulin-dependent proteins as well.

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