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Protein Kinase C Modulates Calcium Sensitivity of Nitric Oxide Synthase in Cerebellar Slices

Daisuke Okada

Laboratory for Neural Networks, Frontier Research Program, Institute of Physical and Chemical Research, RIKEN, Saitama, Japan

Abstract: The possible modulation of nitric oxide (NO) synthase (NOS) activity by protein kinase C (PKC) was investigated. Incubation of rat cerebellar slices with the specific metabotropic glutamate receptor agonist, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylate (*trans*-ACPD) increased cyclic GMP concentration twofold. The increase was dose-dependently blocked by the protein kinase inhibitors staurosporine and calphostin C. Phorbol 12-myristate 13-acetate (PMA), a PKC activator, increased cyclic GMP concentration without glutamate receptor activation. The cyclic GMP increases induced by PMA and *trans*-ACPD were independent of extracellular calcium blocked by N^{ω}-nitro-L-arginine, a specific NOS inhibitor, and were not additive. Measurement of citrulline formation in cerebellar slices confirmed that NOS was activated by *trans*-ACPD and the activation was blocked by calphostin C. These results suggest that metabotropic glutamate receptor activates NOS through PKC. The calcium dependency of NOS activation was assessed in slices incubated with PMA and okadaic acid. NOS in both PMA-treated and untreated slices had similar activities at 100 n*M* free calcium, whereas at 25–70 n*M* free calcium, NOS in PMA-treated slices was more active than that in untreated slices. These results suggest that PKC regulates NO release in resting neurons by modulating the sensitivity of NOS at low calcium concentrations. **Key Words:** Metabotropic glutamate receptor—Nitric oxide synthase—Protein kinase C—Calcium—Calmodulin—Cyclic GMP.
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Neuronal nitric oxide synthase (NOS) converts L-arginine into nitric oxide (NO) and L-citrulline in a calcium/calmodulin (Ca/CM)-dependent manner (Bredt and Snyder, 1990). NO is an activator of soluble guanylyl cyclase, which catalyzes the production of cyclic GMP (cGMP) (Gerzer et al., 1981). In rat cerebellar slices, ionotropic glutamate receptor (iGluR) agonists, such as *N*-methyl-D-aspartate (Garthwaite et al., 1988) or (*R,S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) (Okada, 1992), activate NOS in a manner dependent on calcium influx and subsequently increase cGMP concentration.

Activation of metabotropic glutamate receptor (mGluR) linked to phosphoinositide (PI) turnover also induces cGMP production in rat cerebellar slices through NOS activation (Okada, 1992). A specific mGluR agonist, (±)-1-amino-cyclopentane-*trans*-1,3-dicarboxylate (*trans*-ACPD; Palmer et al., 1989), increased cGMP concentration and this was blocked by N^{ω}-monomethyl-L-arginine, a specific NOS inhibitor. The cGMP increase induced by *trans*-ACPD was reduced after depletion of inositol phospholipids by a long-term treatment with lithium chloride (Berridge et al., 1982) and took place even in a calcium-depleted medium. These previous results suggest a novel mechanism for NOS activation involving PI metabolism triggered by mGluR. Marin et al. (1992) suggested that protein kinase C (PKC), which can be activated by diacylglycerols generated by PI metabolism, contributed to NOS activation by *N*-methyl-D-aspartate in striatal neuronal cultures. Results from in vivo experiments also supported this idea (Hammer et al., 1993). However, it is unclear what property of NOS is modulated by PKC in these studies.

In the present report, the possible involvement of PKC in NOS activation by *trans*-ACPD and the mechanism of NOS activation by PKC was studied using the following three NOS assay methods. First, cGMP accumulation as a result of guanylyl cyclase activation by NO was measured by radioimmunoassay. Second,

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Address correspondence and reprint requests to Dr. D. Okada at Laboratory for Neural Networks, Frontier Research Program, Institute of Physical and Chemical Research, RIKEN, 2-1, Hirosawa, Wako, Saitama 351-01, Japan.

Abbreviations used: *trans*-ACPD, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylate; AMPA, (*R,S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; AP5, 2-amino-5-phosphonovalerate; $[Ca]_{free}$, free calcium concentration; cGMP, cyclic GMP; CM, calmodulin; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; DMSO, dimethyl sulfoxide; iGluR, ionotropic glutamate receptor; MCPG, (*R,S*)-α-methyl-4-carboxyphenylglycine; mGluR, metabotropic glutamate receptor; NO, nitric oxide; NOS, nitric oxide synthase; OA, okadaic acid; PI, phosphoinositide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulfonyl fluoride.

citrulline formation due to NOS activation by *trans*-ACPD was measured in cerebellar slices preloaded with radioactive L-arginine. In the third method to study the effect of PKC on the calcium sensitivity of NOS activity, cerebellar slices were incubated with a PKC activator and then homogenized under conditions suppressing both phosphorylation and dephosphorylation. NOS activity in the homogenate was assayed by determining citrulline formation at controlled concentrations of calcium ions. The results indicated that PKC was involved in NOS activation by *trans*-ACPD and that PKC modulated the calcium sensitivity of NOS.

EXPERIMENTAL PROCEDURES

Rat cerebellar slices

Parasagittal slices of the cerebellar vermis (400 μm thick, ~15 mg wet weight) were prepared from male Wistar-ST rats (6–9 weeks old) in ice-cold Krebs–bicarbonate buffer at pH 7.4 using a vibrating slicer (Dosaka EM, Japan) after decapitation under diethylether anesthesia. The slices were incubated at 34°C for 90 min in 100 ml of Krebs–bicarbonate buffer. For all subsequent incubations, each slice was incubated at 34°C with 1 ml of Krebs buffer in a glass tube containing various drugs. The Krebs–bicarbonate buffer was composed of (m*M*) NaCl 120, KCl 2.0, $MgCl_2$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2, $NaHCO_3$ 26, and glucose 11, and continuously and gently supplied with 5% CO_2 + 95% O_2.

cGMP measurement

The cGMP content of cerebellar slices was assayed by radioimmunoassay (Amersham, U.K.) as described previously (Okada, 1992). Incubation of slices with *trans*-ACPD (0.1–1 m*M*, 5 min) or phorbol 12-myristate 13-acetate (PMA; 30 n*M* to 10 μ*M*, 10 min) was done in the presence of 3-isobutyl-1-methylxanthine (0.1 m*M*), which partially suppresses phosphodiesterase activities. This was necessary to detect the agonist-induced cGMP increase but did not affect the basal cGMP level (Okada, 1992) or phospholipase activities (Hong, 1983).

NOS assay in cerebellar slices

Slices were incubated in Krebs buffer containing 6×10^6 cpm of L-[^{3}H]arginine (2.29 TBq/mmol, Amersham) for 45 min and then with 1 m*M* *trans*-ACPD for 5 min. Slices were homogenized in 20 μl of 20% ice-cold trichloroacetic acid and the homogenate was centrifuged. After extracting trichloroacetic acid with 100 μl of water-saturated diethyl ether four times, the pH of the supernatant was adjusted to 6 with NaOH, and the [^{3}H]citrulline formed was quantified.

NOS assay in vitro

Slices were incubated with okadaic acid (OA, 1 μ*M*) for 10 min and then with PMA (30–300 n*M*) for another 10 min. Slices were then homogenized with a Polytron-type homogenizer in 400 μl of 50 m*M* HEPES-NaOH buffer at pH 7.4 containing 1 m*M* EGTA, proteinase inhibitors [1 m*M* EDTA, 10 μg/ml leupeptin, 1.4 μ*M* pepstatin A, and 1 m*M* phenylmethanesulfonyl fluoride (PMSF)], 1 μ*M* OA, and 0.1 μ*M* staurosporine. Ten microliters of the homogenate was added to 10 μl of NOS reaction buffer and incubated at 25°C for 15 min. NOS reaction buffer is composed of (final concentrations) 50 m*M* HEPES-NaOH buffer, pH 7.4, NOS activators (3 μ*M* tetrahydrobiopterin and 0.1 μ*M* CM), NOS substrates (1 m*M* NADPH, 1 μ*M* L-arginine, and 4 $\times 10^5$ cpm L-[^{3}H]arginine), and 0.1 mg/ml heparin. The reaction was terminated by the addition of 5 μl of ice-cold 100% trichloroacetic acid and then treated as described in the previous section.

The important feature of this in vitro NOS assay is that the stimulation of PKC was performed in slices, which conserves physiological compartmentalization, thus reducing nonphysiological phosphorylation and NOS denaturation. During the NOS assay of the slice homogenates, changes in protein phosphorylation should have been suppressed by the addition of staurosporine and protein phosphatase inhibitors to the homogenization and incubation media. Staurosporine, at 50 n*M*, is a nonspecific protein kinase inhibitor and inhibits not only PKC but also CM-dependent protein kinase II, which may affect NOS activity (Nakane et al., 1991). Protein phosphatase types I and IIa were suppressed by OA, type IIb (calcineurin) by EGTA, and type IIc (requires Mg) by EDTA (Cohen, 1991). Dawson et al. (1993) suggested that calcineurin dephosphorylated NOS, resulting in its activation. In the in vitro NOS assay performed in the present study, NOS was assayed at free calcium concentrations <100 n*M*, where calcineurin has little activity (Li, 1984). Two possible major mechanisms of calcium release were suppressed in the NOS assay medium to exclude their influence on the possible modulation of calcium release mechanisms by PKC. Heparin was used to block inositol trisphosphate–sensitive calcium release (Worley et al., 1987), and the formation of cyclic ADP-ribose, a possible endogenous agonist of the ryanodine-sensitive calcium release, was inhibited by staurosporine (Galione et al., 1993).

Estimation of free calcium concentrations

Free calcium concentrations in the in vitro NOS assay at pH 7.4 were estimated using stability constants of metal–chelator and nucleotide–metal complexes and their pK_a values (Dawson et al., 1986). The stability constants used (log values) were EDTA-Ca 10.69, EGTA-Ca 11.0, EGTAH-Ca 5.3, Mg-ATP 4.3, Ca-ATP 3.8, Mg-ADP 3.3, Ca-ADP 2.9. The pK_a values were 10.24, 6.16, 2.66, and 2.0 for EDTA, 9.5, 8.8, and 2.7 for EGTA, 6.5 and 4.1 for ATP, and 6.4 and 3.9 for ADP. The volume of a slice was estimated to be 20 μl, assuming a slice to be a cylinder 0.4 mm in height, whose bases are circles with 4-mm radii. Total Mg^{2+} concentration in a slice was assumed to be 1 m*M*. The total concentrations of ATP and ADP in a slice were measured by HPLC (Toso, Japan) and estimated to be 250 and 100 μ*M*, respectively. Nucleotides were separated with a TSK-DEAE-2SW column (4.6 i.d. × 250 mm, Toso) with 80 m*M* potassium phosphate buffer at pH 7.0, containing 0.1 m*M* EDTA and 20% acetonitrile as the mobile phase, with a flow rate of 0.8 ml/min, and detected by absorption at 259 nm. Peaks corresponding to ATP (25.3 min) and ADP (15.6 min) were confirmed by internal standards and elimination by hexokinase (Sigma) and were without contaminants.

Separation of citrulline from arginine

Citrulline was separated from arginine by TLC. The supernatants after neutralization, together with nonradioactive L-citrulline and L-arginine as internal standards, were spotted onto a cellulose TLC plate (Funacell SF, Funakoshi Co., Japan). The total volume applied was <5 μl. The mobile phase was aqueous phenol/ethanol/ammonia/water (10:12:1:4, by volume). The citrulline band, visualized by ninhydrin, was scraped and its radioactivity was counted. Radioac-

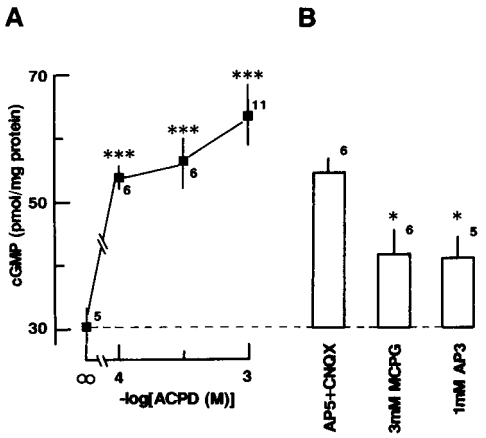

FIG. 1. A: cGMP concentrations after incubation of cerebellar slices with the indicated concentrations of *trans*-ACPD for 5 min. The basal level is indicated by a broken line. The numbers of experiments are shown beside the squares. Asterisks indicate significant increases from the basal cGMP level determined by Student's *t* test (***$p < 0.1\%$, *$p < 5\%$). Vertical bars, SEM. **B:** Effect of AP5+CNQX, MCPG, and AP3 on the *trans*-ACPD-induced increase in cGMP.

tivity in the remaining part of the lane was also counted. The citrulline count was divided by the summation of citrulline and arginine counts and then multiplied by applied radioactivity, which was assumed to be 15,000 cpm for NOS assay in slices and 26,000 cpm for in vitro NOS assay. This normalization excludes error caused by difference in the application volume. The recovery was usually >95%. To confirm the complete separation, one lane was divided into 20 segments 5 mm long and the radioactivity in each part was counted. The only two major radioactive materials, corresponding to citrulline and arginine, were found as separate bands. Two-dimensional TLC, using *n*-butanol/acetic acid/water (12:3:5) as the second mobile phase, confirmed that the presumed citrulline spot contained only citrulline. No radioactivity was retained at the origin.

Materials

Materials were purchased from companies as follows: *trans*-ACPD, 2-amino-5-phosphonovalerate (AP5), and (*R,S*)-α-methyl-4-carboxyphenylglycine (MCPG) (Tocris Neuramine, U.K.); AMPA and tetrahydrobiopterin (RBI, U.S.A.); HEPES, EDTA, and EGTA (Wako-Dojindo, Japan); W7, W13, and CM (Seikagaku Kogyo, Japan); calphostin C (Kyowa Medex, Japan); PMA, 4α-phorbol, leupeptin, pepstatin A, PMSF, heparin, staurosporine, N^{ω}-nitro-L-arginine, NADPH, 3-isobutyl-1-methylxanthine, and dimethyl sulfoxide (DMSO) (Sigma, U.S.A.). OA and other chemicals were from Wako Pure Chemicals, Japan.

RESULTS

PKC increases cGMP concentration in cerebellar slices

The conclusion of the previous report (Okada, 1992) that *trans*-ACPD increased cGMP concentration through a mechanism mediated by mGluR was confirmed. The cGMP concentration level in cerebellar slices after incubation with 100 μ*M trans*-ACPD was 53.5 ± 5.5 pmol/mg of protein (mean ± SEM, n = 7), whereas the basal level was 30.4 ± 3.0 pmol/mg of protein (n = 5), as shown in Fig. 1. N^{ω}-Nitro-L-arginine (30 μ*M*, 10 min), a specific inhibitor of NOS (Lambert et al., 1991), eliminated the cGMP increase by 0.3 m*M trans*-ACPD (24.5 ± 4.1 pmol/mg of protein, n = 8). Increases in cGMP induced by *trans*-ACPD were not inhibited by 10-min incubation with iGluR antagonists AP5 (200 μ*M*) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 100 μ*M*) (54.4 ± 2.8 pmol/mg of protein, n = 6), whereas a specific mGluR antagonist, MCPG (3 m*M*, 20 min; Eaton et al., 1993), significantly reduced the cGMP increase by 100 μ*M trans*-ACPD (44.8 ± 4.0 pmol/mg of protein, n = 4, $p < 5\%$). These results excluded the involvement of iGluR in this system such as possible enhancement of iGluR responses by *trans*-ACPD or iGluR activation by endogenous glutamate released by the *trans*-ACPD application.

Involvement of PKC in the mGluR-mediated cGMP increase was studied using protein kinase inhibitors. As shown in Fig. 2A, staurosporine and calphostin C dose-dependently inhibited the effect of *trans*-ACPD on cGMP concentration with the half-inhibition concentrations of ~3 and ~30 n*M*, respectively. These

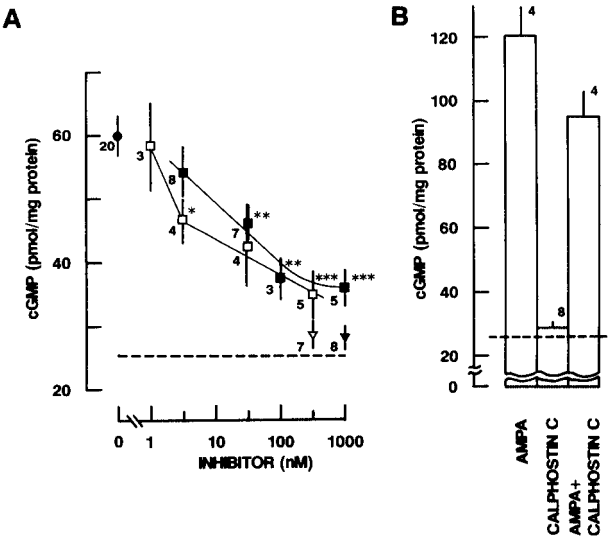

FIG. 2. A: Dose-dependent inhibition by protein kinase inhibitors of the cGMP increase induced by *trans*-ACPD (300 μ*M*, 5 min) in cerebellar slices. Before addition of *trans*-ACPD, slices were incubated with either staurosporine (open squares) or calphostin C (closed squares) for 45 min. A closed circle indicates cGMP concentration in slices incubated with *trans*-ACPD in the presence of DMSO (0.1%). Open and closed triangles indicate cGMP concentration in slices incubated with staurosporine and calphostin C alone, respectively. Asterisks show significant inhibition (***$p < 0.1\%$, **$p < 1\%$, *$p < 5\%$). **B:** Effect of AMPA (10 μ*M*, 5 min) and calphostin C (1 μ*M*) on the cGMP concentration in cerebellar slices. The basal cGMP concentration in slices incubated with DMSO alone is indicated by broken lines in both A and B.

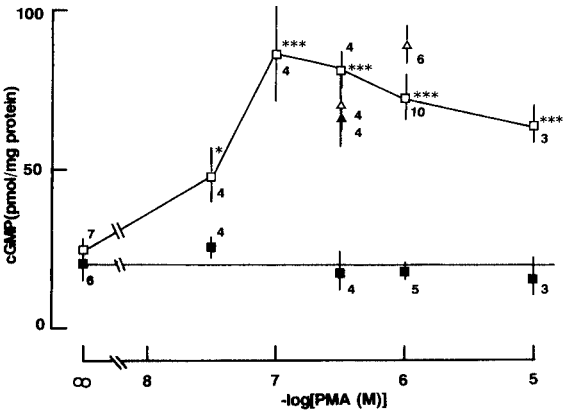

FIG. 3. The effect of PMA on cGMP concentration in cerebellar slices. Open squares indicate cGMP concentration after incubation with PMA for 10 min. Closed squares indicate the effect of PMA in the presence of N^{ω}-nitro-L-arginine (30 μM, 10 min). Open triangles indicate the effect of PMA in a calcium-depleted medium (Krebs–bicarbonate buffer containing 0.2 mM EGTA instead of $CaCl_2$, 15 min). The closed triangle indicates the effect of PMA in the presence of AP5 (100 μM, 10 min), CNQX (10 μM, 10 min), and MCPG (3 mM, 20 min). Asterisks show significant increase from the cGMP concentration at 0 M PMA (***p < 0.1%, *p < 5%).

values were similar to their IC_{50} values reported for PKC inhibition, i.e., 5 nM for staurosporine (Tamaoki et al., 1986) and 50 nM for calphostin C (Kobayashi et al., 1989). Although calphostins, at micromolar concentrations, may modify a variety of molecules through membrane damage as reported by Wang et al. (1993), calphostin C, at lower concentrations, inhibits PKC activity specifically (Kobayashi et al., 1989). In the present study, calphostin C at concentrations as low as 30 nM significantly inhibited the cGMP increase by *trans*-ACPD (Fig. 2A). Furthermore, and in marked contrast, calphostin C did not affect cGMP increases evoked by AMPA even at 1 μM (Fig. 2B), excluding possible nonspecific actions of calphostin C. These results suggest that PKC is necessary for the cGMP increase induced by *trans*-ACPD but not by AMPA. DMSO (25.4 ± 2.5 pmol/mg of protein, n = 7) or inhibitors alone did not alter the basal cGMP levels.

PMA, a potent PKC activator (Castagna et al., 1982), dose-dependently and consistently increased cGMP concentration in cerebellar slices as shown in Fig. 3. N^{ω}-Nitro-L-arginine completely suppressed this increase, without affecting basal cGMP levels. This implies that PMA increases the cGMP concentration through NOS activation. The following experiments indicated that PMA mimics the effects of *trans*-ACPD in increasing the cGMP concentration. (1) The PMA-induced cGMP increase was not significantly suppressed by a mixture of glutamate antagonists composed of AP5, CNQX, and MCPG (filled triangle in Fig. 3); therefore, the PMA effect is independent of glutamatergic transmission. (2) PMA, like *trans*-ACPD (Okada, 1992), increased cGMP concentration of the slices in a calcium-depleted medium (open triangles in Fig. 3). (3) The cGMP concentration after coapplication of *trans*-ACPD (1 mM) and PMA (1 μM) was 55.3 ± 8.0 pmol/mg of protein (n = 6), which was similar to the cGMP level achieved by applications of *trans*-ACPD or PMA alone, suggesting that *trans*-ACPD and PMA act via the same pathway. The results obtained by PKC activation and protein kinase inhibition (Figs. 2 and 3) suggest that *trans*-ACPD increases cGMP concentration through PKC activation.

NOS activation by *trans*-ACPD requires PKC

NOS activation by *trans*-ACPD was assayed in cerebellar slices preloaded with L-[^{3}H]arginine. In unstimulated slices, the L-[^{3}H]citrulline count was 969 ± 69 cpm (n = 5). The count increased to 1,325 ± 123 cpm (n = 5) after incubation with 1 mM *trans*-ACPD, indicating NOS activation by *trans*-ACPD. This increase was 1.37-fold and significant (p < 5%) as shown in Fig. 4. Figure 4 also shows that calphostin C at 1 μM completely prevented NOS activation by *trans*-ACPD, confirming that PKC was necessary for NOS activation through mGluR. Also, CM antagonists, W13 and W7 at 100 μM, suppressed NOS activation by *trans*-ACPD (data not shown). This result can be explained by the following two different possibilities, which cannot be discriminated by using slices: CM is involved in NOS activation by *trans*-ACPD and CM is required for NOS activity itself. Further studies are necessary for discriminating these possibilities.

PKC shifts calcium sensitivity of NOS in vitro

NOS activities in homogenates of slices treated with PMA + OA and DMSO were compared at various free calcium concentrations. The results are shown in Fig. 5A. When the calcium concentration of the NOS reaction buffer was adjusted to 830 μM, which corresponded to a free calcium concentration ($[Ca]_{free}$) of

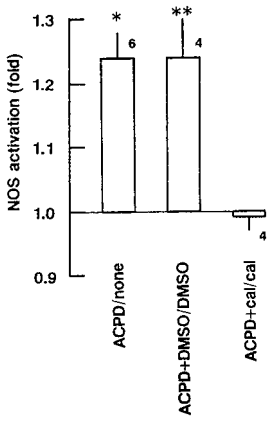

FIG. 4. NOS activation by *trans*-ACPD in cerebellar slices preloaded with L-[^{3}H]arginine. Slices were incubated with L-[^{3}H]arginine for 45 min and then with *trans*-ACPD at 1 mM for 5 min. DMSO (0.1%) or calphostin C (1 μM) was added to the incubation medium simultaneously with L-[^{3}H]-arginine. The ordinate shows the ratio of the normalized citrulline radioactivities between slices incubated with and without *trans*-ACPD. For example, the column denoted (ACPD + DMSO)/DMSO indicates the ratio of normalized citrulline radioactivities between slices incubated with ACPD + DMSO and DMSO. The total radioactivity in a slice was not changed significantly by incubation with those compounds described here. Calphostin C is abbreviated to cal. Asterisks show the significant activation by *trans*-ACPD (*p < 5%, **p < 1%).

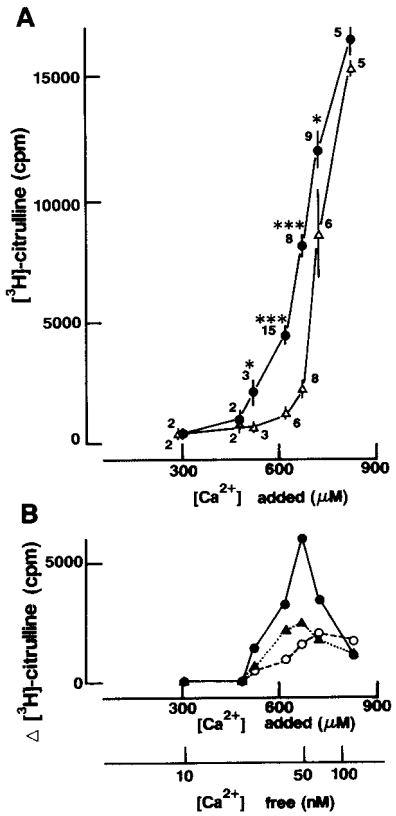

FIG. 5. A: Calcium dependency of NOS activity in homogenates prepared from PMA-treated and untreated slices. Cerebellar slices were incubated with 1 μM OA for 10 min and then with 0.3 μM PMA for another 10 min (PMA treated, closed circles) or with 0.13% of DMSO for 20 min (untreated, open triangles). NOS activities in the homogenates of these slices were assayed at various calcium concentrations indicated on the abscissa. $[Ca^{2+}]_{added}$ indicates calcium concentrations added in the NOS reaction buffer. Asterisks show significant increases in the PMA-treated samples compared with untreated samples at the same calcium concentrations (***$p < 0.1\%$, *$p < 5\%$). **B:** Increases in NOS activities in slices treated with PMA and OA. The difference in NOS activities between PMA-treated and untreated slices (closed circles) were calculated from the data illustrated in A and plotted. The similar differences in the NOS activities of slices treated with PMA alone (open circles) or OA alone (closed triangles) are also shown. The ordinate shows the activity differences. The abscissa $[Ca^{2+}]_{added}$ is the same as that in A. The $[Ca^{2+}]_{free}$ scale represents free calcium concentration and is applicable to both A and B.

100 nM, NOS activity in both PMA-treated (16,224 ± 546 cpm, n = 5) and untreated (15,548 ± 676 cpm, n = 5) slices was almost identical. In contrast, at $[Ca]_{free}$ between 25 and 70 nM, higher NOS activities were observed in PMA-treated slices (at $[Ca]_{free}$ 50 nM, 8,502 ± 416 cpm, n = 6) than in untreated slices (2,158 ± 312 cpm, n = 6). Figure 5B shows the difference in NOS activities between PMA-treated and untreated slices. The maximum increase was about fourfold at 50 nM $[Ca]_{free}$. NOS activity was only moderately enhanced by treatment with OA or PMA alone (Fig. 5B). Thus, incubation of slices with PMA + OA shifted the calcium sensitivity of NOS activity so that NOS was active at lower calcium concentrations.

Figure 6A shows NOS activity at 50 nM $[Ca]_{free}$ in slices treated with various concentrations of PMA in the presence of 1 μM OA. PMA applied at concentrations >30 nM significantly increased NOS activity in slices compared with OA application alone. An inactive phorbol, 4α-phorbol, applied instead of PMA did not have any significant effect compared with OA application alone. Therefore, the effect of PMA is most likely to be due to PKC activation. Figure 6B shows that incubation of slices with PMA and OA in the presence of staurosporine completely suppressed the enhancement of NOS activity. Therefore, calcium sensitivity of NOS was altered by protein kinase activity.

Figure 7 shows that submicromolar concentrations of N^{ω}-nitro-L-arginine to the NOS reaction buffer simi-

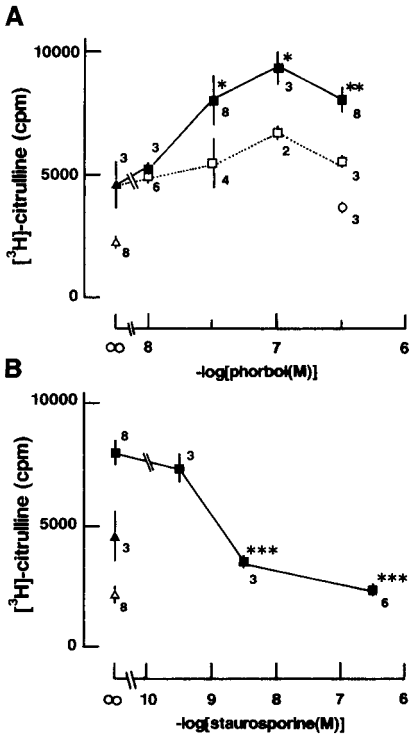

FIG. 6. Involvement of PKC in NOS activation induced by PMA + OA treatment. NOS activity was assayed at $[Ca]_{free}$ = 50 nM. **A:** The relationship between concentration of phorbol esters and NOS activation. Cerebellar slices were incubated in the medium containing indicated concentrations of PMA and 1 μM OA (closed squares), indicated concentrations of 4α-phorbol and 1 μM OA (open squares), 0.3 μM PMA alone (open circle), 1 μM OA alone (closed triangle), or DMSO (open triangle). Asterisks shows significant increases compared with OA alone (*$p < 5\%$, **$p < 1\%$). **B:** Inhibition by staurosporine of NOS activation by PMA. Cerebellar slices were incubated with the medium containing 0.3 μM PMA and 1 μM OA in the presence of indicated concentrations of staurosporine (closed squares), 1 μM OA alone (closed triangle), or DMSO alone (open triangle). ***Significant inhibition ($p < 0.1\%$).

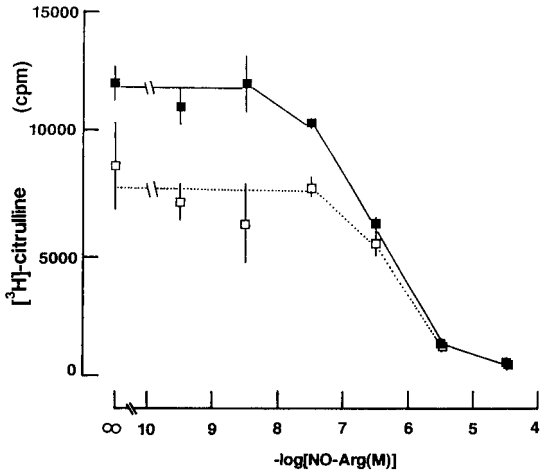

FIG. 7. Inhibition of NOS in PMA-treated or untreated slices by N^{ω}-nitro-L-arginine. Cerebellar slices were incubated with the medium containing 0.3 μM PMA and 1 μM OA (closed squares) or DMSO (open squares). NOS was assayed at $[Ca]_{free}$ = 70 nM in the presence of indicated concentrations of N^{ω}-nitro-L-arginine. Each symbol represent a mean of three assays with an SEM bar.

larly suppressed NOS activity in both PMA-treated and untreated slices. N^{ω}-Nitro-L-arginine inhibits neuronal NOS at submicromolar concentrations, whereas >100 μM is necessary for the inhibition of inducible NOS (Lambert et al., 1991). Therefore, this result suggests that inducible NOS, which does not require calcium for activation, is not involved in the shift of NOS calcium sensitivity.

DISCUSSION

Two pathways of NOS activation by glutamate receptors

The present study indicates that mGluR activates NOS through a PKC-mediated mechanism in cerebellar slices (Figs. 2–4). In a previous report (Okada, 1992), it was suggested that *trans*-ACPD activated NOS through PI metabolism, which in turn can enhance both intracellular calcium and PKC activity. PKC translocation after incubation of cerebellar slices with *trans*-ACPD was observed (D. Okada, unpublished observation). However, *trans*-ACPD also raises intracellular calcium levels, for example, in Purkinje cells, to concentrations of several hundred nanomolar through a Na/Ca exchanger–mediated mechanism (Vranesic et al., 1991) or by release from intracellular stores sensitive to inositol trisphosphate (Llano et al., 1991). Because NOS is likely to be active at $[Ca]_{free}$ > 100 nM, the possibility that mGluR, as well as iGluR, can activate NOS after calcium increase cannot be excluded. However, the contribution of this mechanism may be small and that of PKC-mediated mechanism may be predominant, because cGMP accumulation induced by *trans*-ACPD was small in the presence of PKC inhibitors (Fig. 2A).

The NOS assays under controlled calcium concentrations suggest that PKC enhances NOS activity at free calcium concentrations as low as 20–70 nM (Fig. 5) due to protein phosphorylation by PKC (Fig. 6). This level of $[Ca]_{free}$ may be appropriate for resting neurons, because Konnerth et al. (1992), for example, observed $[Ca]_{free}$ to be <100 nM in all resting Purkinje cells. Therefore, mGluR may regulate NO release in resting cells in the cerebellum through the PKC-regulated mechanism, whereas NOS activation by iGluR may occur after transient intracellular calcium increase due to neuronal excitation.

PKC activity resulting from mGluR activation can last longer than calcium increases (Nishizuka, 1992), due to prolonged production of diacylglycerol by phospholipase D activity (Martinson et al., 1990) or to proteolytic release of the active PKC fragment (Inoue et al., 1977). Such observations suggest that PKC can keep NOS active after transient elevations in calcium concentration after iGluR-mediated actions of glutamate have subsided. If a subsequent stimulus comes in time, this long-lasting basal NO release could superimpose on the NO release induced by the second stimulus. Thus, the two mechanisms of NO release may in concert result in higher concentrations of NO. This mechanism would depend on the temporal pattern of stimuli and may relate to the observation that NO sensitivity of hippocampal long-term potentiation depended on temporal pattern of stimuli or the stimulus intensity (Zorumski and Izumi, 1993).

Galione et al. (1993) reported that NO or cGMP slowly stimulated the conversion of NAD^+ into cyclic ADP-ribose, an endogenous agonist of ryanodine-sensitive calcium release, via protein phosphorylation. Casabona et al. (1993) suggested the possibility that mGluR triggers the synthesis of cyclic ADP-ribose in hippocampal membranes. These observations, taken together with the present result, suggest that NOS activation by mGluR may also contribute to the synthesis of cyclic ADP-ribose, adding another mechanism to calcium dynamics.

The PKC-substrate protein in NOS regulation

The PKC-substrate protein that is responsible for NOS activation is not yet known and is an interesting subject for further study. The most plausible substrate is NOS itself. NOS activation by PKC has been reported in neuronal culture (Marin et al., 1992) or in vivo (Hammer et al., 1993); however, in in vitro experiments, it is still an open question whether PKC activates purified brain NOS or not. Bredt et al. (1992) observed NOS inhibition by PKC, whereas Nakane et al. (1991) reported NOS activation by PKC. In the report by Brüne and Lapetina (1991), PKC did not phosphorylate NOS. These apparent contradictions could be attributed to different phosphorylation sites under the various experimental conditions or to instability of the purified NOS. Another possibility is that an as yet unknown protein regulates NOS activity. Al-

though a protein regulating calcium sensitivity of NOS through phosphorylation by PKC has not been reported, cytosolic proteins that stabilized NOS were found in rat peritoneal polymorphonuclear neutrophils (Kosuga et al., 1990), suggesting the existence of NOS-regulating proteins.

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REFERENCES

Berridge M. J., Downes C. P., and Hanley M. R. (1982) Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.* **206,** 587–595.

Bredt D. S. and Snyder S. H. (1990) Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA* **87,** 682–685.

Bredt D. S., Ferris C. D., and Snyder S. H. (1992) Nitric oxide synthase regulatory sites. *J. Biol. Chem.* **267,** 10976–10981.

Brüne B. and Lapetina E. G. (1991) Phosphorylation of nitric oxide synthase by protein kinase A. *Biochem. Biophys. Res. Commun.* **181,** 921–926.

Casabona G., Sturiale L., Fazzio A., Sarpietro M. G., Genazani A. A., Impallomeni G., Garozzo D., Cambria A., and Nicoletti F. (1993) Hippocampal membranes convert NAD^+ into cyclic ADP-ribose and ADP-ribose: a model to study the interaction between metabotropic glutamate receptors and the cyclic-ADP-ribose-generating system. *Funct. Neurol.* (Suppl. 4), 14.

Castagna M., Takai Y., Kaibuchi K., Sano K., Kikkawa U., and Nishizuka Y. (1982) Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257,** 7847–7851.

Cohen P. (1991) Classification of protein-serine/threonine phosphatases: identification and quantitation in cell extracts. *Methods Enzymol.* **201,** 469–476.

Dawson, R. M. C., Elliot D. C., Elliot W. H., and Jones K. M. (1986) Stability constants of metal complexes, in *Data for Biochemical Research*, 3rd edit., pp. 399–415. Oxford University Press, New York.

Dawson T. M., Steiner J. P., Dawson V. L., Dinerman J. L., Uhl G. R., and Snyder S. H. (1993) Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. *Proc. Natl. Acad. Sci. USA* **90,** 9808–9812.

Eaton S. A., Jane D. E., Jones P. L. St. J., Porter R. H. P., Pook P. C.-K., Sunter D. C., Udvarhelyi P. M., Roberts P., Salt T. E., and Watkins J. C. (1993) Competitive antagonism at metabotropic glutamate receptors by (*S*)-4-carboxyphenylglycine and (*R,S*)-alpha-methyl-4-carboxyphenylglycine. *Eur. J. Pharmacol.* **244,** 195–197.

Galione A., White A., Willmott N., Turner M., Potter B. V. L., and Watson S. P. (1993) cGMP mobilizes intracellular Ca^{2+} in sea urchin eggs by stimulating cyclic ADP-ribose synthesis. *Nature* **365,** 456–459.

Garthwaite J., Charles S. L., and Chess-Williams R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* **336,** 385–388.

Gerzer R., Hofman F., and Schultz G. (1981) Purification of a soluble, sodium-nitroprusside-stimulated guanylyl cyclase from bovine lung. *Eur. J. Biochem.* **116,** 476–486.

Hammer B., Parker W. D. Jr., and Bennett J. P. Jr. (1993) NMDA receptors increase OH radicals *in vivo* by using nitric oxide synthase and protein kinase C. *Neuroreport* **5,** 72–74.

Hong S. L. (1983) Inhibition of prostacyclin synthesis in endothelial cells by methylisobutylxanthine is not mediated through elevated cAMP level. *Biochim. Biophys. Acta* **754,** 258–263.

Inoue M., Kishimoto A., Takai Y., and Nishizuka Y. (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. *J. Biol. Chem.* **252,** 7610–7616.

Kobayashi E., Nakano H., Morimoto M., and Tamaoki T. (1989) Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **159,** 548–553.

Konnerth A., Dreessen J., and Augustine G. (1992) Brief dendritic calcium signals initiate long-lasting synaptic depression in cerebellar Purkinje cells. *Proc. Natl. Acad. Sci. USA* **89,** 7051–7055.

Kosuga K., Yui Y., Hattori R., Eizawa H., Hiki K., and Kawai C. (1990) Stabilizing factors of nitric oxide synthase. *Biochem. Biophys. Res. Commun.* **172,** 705–708.

Lambert L. E., Whitten J. P., Baron B. M., Cheng H. C., Doherty N. S., and McDonald I. A. (1991) Nitric oxide synthesis in the CNS, endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci.* **48,** 69–75.

Li H.-C. (1984) Activation of brain calcineurin phosphatase towards nonprotein phosphoesters by Ca^{2+}, calmodulin, and Mg^{2+}. *J. Biol. Chem.* **259,** 8801–8807.

Llano I., Dreessen J., Kano M., and Konnerth A. (1991) Intradendritic release of calcium induced by glutamate in cerebellar Purkinje cells. *Neuron* **7,** 577–583.

Marin P., Lafon-Cazal M., and Bockaert J. (1992) A nitric oxide synthase activity selectively stimulated by NMDA receptors depends on protein kinase C activation in mouse striatal neurons. *Eur. J. Neurosci.* **4,** 425–432.

Martinson E. A., Trilivas I., and Brown J. H. (1990) Rapid protein kinase C-dependent activation of phospholipase D leads to delayed 1,2-diglyceride accumulation. *J. Biol. Chem.* **265,** 22282–22287.

Nakane M., Mitchell J., Förstermann U., and Murad F. (1991) Phosphorylation by calcium calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase. *Biochem. Biophys. Res. Commun.* **180,** 1396–1402.

Nishizuka Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258,** 607–614.

Okada D. (1992) Two pathways of cyclic GMP production through glutamate receptor-mediated nitric oxide synthesis. *J. Neurochem.* **59,** 1203–1209.

Palmer E., Monaghan D. T., and Cotman C. W. (1989) *trans*-ACPD, a selective agonist of the phosphoinositide-coupled excitatory amino acid receptor. *Eur. J. Pharmacol.* **166,** 585–587.

Tamaoki T., Nomoto H., Takahashi I., Kato Y., Morimoto M., and Tomita F. (1986) Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135,** 397–402.

Vranesic I., Batchelor A., Gähwiler B. H., Garthwaite J., Staub C., and Knöpfel T. (1991) *trans*-ACPD-induced Ca^{2+} signals in cerebellar Purkinje cells. *Neuroreport* **2,** 759–762.

Wang S. S.-H., Mathes C., and Thompson S. H. (1993) Membrane toxicity of the protein kinase C inhibitor calphostin A by a free-radical mechanism. *Neurosci. Lett.* **157,** 25–28.

Worley P. F., Baraban J. M., Supattapone S., Wilson V. S., and Snyder S. H. (1987) Characterization of inositol trisphosphate receptor binding in brain. *J. Biol. Chem.* **262,** 12132–12136.

Zorumski C. F. and Izumi Y. (1993) Nitric oxide and hippocampal synaptic plasticity. *Biochem. Pharmacol.* **46,** 777–785.