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Inhibition of Neuronal Nitric Oxide Synthase Activity by 3-[2-[4-(3-Chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole Dihydrochloride 3.5 Hydrate (DY-9760e), a Novel Neuroprotective Agent, *In Vitro* and in Cultured Neuroblastoma Cells *In Situ*

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ABSTRACT. DY-9760e, 3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate, a novel calmodulin (CaM) antagonist, possesses neuroprotective activity. In the current study, we examined the effects of DY-9760e on nitric oxide synthase (NOS) activities *in vitro* and on calcium ionophore-induced NO production *in situ*. DY-9760e inhibited both neuronal NOS and endothelial NOS activities without affecting inducible NOS activity. It also inhibited purified neuronal NOS activity with a potency similar to that seen for purified CaM kinase II activity *in vitro*. Furthermore, DY-9760e significantly inhibited Ca^{2+} ionophore (A23187)-induced NO production in mouse N1E-115 neuroblastoma cells, at a concentration of less than 1 μM. In contrast, no apparent inhibitory effect on Ca^{2+}/CaM-dependent protein kinase II activity was observed in cultured hippocampal neurons up to 5 μM. These results suggest that the inhibitory effect of DY-9760e on CaM-dependent NOS activities underlies neuroprotective effects of the agent. BIOCHEM PHARMACOL **60**;5:693–699, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. calmodulin antagonist; brain ischemia; nitric oxide synthase; CaM kinase II; neuroprotection

CaM§ antagonists and inhibitors for Ca^{2+}/CaM-dependent enzymes have been known to have neuroprotective effects on glutamate neurotoxicity and ischemic brain damage. For example, CaM antagonists such as W-7 and calmidazolium have neuroprotective effects on NMDA-induced neuronal cell death [1]. FK506, an immunosuppressant, inhibits Ca^{2+}/CaM-dependent phosphatase (calcineurin) activity following binding to FK506-binding proteins (FKBPs) and thereby exerts neuroprotective actions in experimental models of stroke [2, 3] and in glutamate neurotoxicity [1]. In addition, KN62, an inhibitor of CaM kinase II, provides neuroprotection against NMDA- and hypoxia/hypoglycemia-induced cell death [4]. We recently characterized a new neuroprotective drug, DY-9760e (3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate), which inhibits various CaM-dependent enzymes [5] and exerts a powerful neuroprotective action in transient focal cerebral ischemia [6]. Among the CaM-dependent enzymes tested, DY-9760e more selectively inhibited CaM kinase II, calcineurin, and CaM-dependent phosphodiesterase with K_i values of 1.4, 2.0, and 3.8 μM, respectively. In contrast, a higher concentration of DY-9760e was required to inhibit CaM kinase IV and myosin light chain kinase (K_i = 12 and 133 μM, respectively). DY-9760e could bind directly to CaM, and the inhibition of CaM-dependent enzymes was competitive against CaM [5]. The cytoprotective effect of DY-9760e against calcium ionophore-induced cell injury was also evident in N1E-115 cells, a neuroblastoma cell line [5]. N1E-115 cells are well-established models of neuronal differentiation [7] that express neuronal NOS (nNOS) [8]. In an experimental model of transient focal cerebral ischemia, intravenous injection of DY-9760e at a dose of 1 mg kg^{-1} hr^{-1} for 6 hr significantly reduced infarct volume by 60%, even when given 1 hr after ischemia [6]. Although DY-9760e may be a unique thera-

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§ *Abbreviations*: CaM, calmodulin; NMDA, *N*-methyl-d-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; HUVEC, human umbilical vein endothelial cells; CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; DTT, dithiothreitol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; L-NAME, L-*n*-nitro-L-arginine methyl ester; and MBP, myelin basic protein.

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peutic agent for acute ischemic brain damage, the molecular mechanism underlying the neuroprotective effect has not been elucidated.

NO is generated by NOS, which is a Ca^{2+}/CaM-dependent enzyme. The production of NO has been implicated in neuronal injury after ischemia, trauma, and numerous neurodegenerative disorders [9, 10]. The combination of NO with superoxide forms a stronger and more toxic oxidant, peroxynitrite ($ONOO^-$) [10]. Excessive stimulation of glutamate receptors may induce neuron death by production of both NO [1, 11] and superoxide [12, 13]. We therefore investigated whether the new CaM antagonist DY-9760e inhibits NOS activity *in vitro* and *in situ*. We found that DY-9760e inhibited nNOS and endothelial NOS (eNOS) but not inducible NOS (iNOS), and also reduced NO production in N1E-115 cells. These results support the idea that DY-9760e elicits a neuroprotective effect by inhibiting NOS activity in transient focal brain ischemia.

MATERIALS AND METHODS

Reagents

DY-9760e was synthesized by the Daiichi Pharmaceutical Co. [5]. The following chemicals and reagents were obtained from the indicated sources: L-[2,3-^{3}H]arginine, DuPont-New England Nuclear; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, the Seikagaku Co.; calmodulin, Sigma; and HUVEC, Clonetics. nNOS was purified from rat cerebellum by the method of Bredt and Snyder [14]. The purity of nNOS was judged to be greater than 90% by SDS–PAGE, as shown in Fig. 1. The specific activity of purified nNOS was 260 nmol/mg/min when NOS activity was measured with 10 μM L-arginine as a substrate.

Cell Culture and Preparation of Cell Extract

Neonatal rat hippocampal cell culture was conducted as described [15]. HUVEC were cultured according to the protocol of the supplier (Clonetics). Primary glial cell cultures were prepared by the methods of McCarthy and de Vellis [16]. Microglial cells were collected as free-floating cells in the primary glial cultures [17] and cultivated in astrocyte-conditioned medium. The purity of the cultures was verified by immunostaining with ED1, a microglial cell marker, and was generally >95%. To prepare iNOS, microglial cells were treated with 1 μg/mL of lipopolysaccharide (LPS) for 24 hr. To obtain cell extracts for the NOS assay, microglial cells were scraped from the culture dish and were homogenized by ultrasonication in a solubilization buffer containing 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 1 mM DTT, 0.1 mM leupeptin, 75 μM pepstatin A, and 0.1 mg/mL of aprotinin. To obtain extracts from cerebellum, one rat cerebellum was homogenized in solubilization buffer with a Teflon–glass homogenizer. After centrifugation, an aliquot

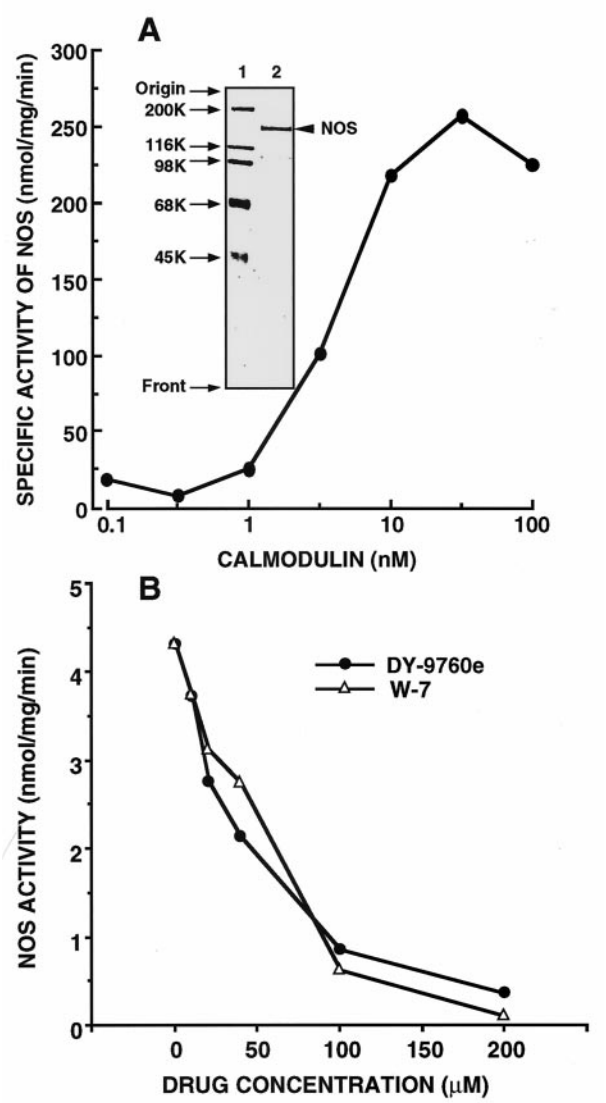

FIG. 1. CaM-dependent activation of purified nNOS and its inhibition by DY-9760e and W-7. (A) NOS purified from rat cerebellum was homogeneous by SDS–PAGE, as shown in the inset, and was activated by increasing CaM concentrations. (B) In the presence of 10 nM CaM, NOS activity was assayed in the presence of various concentrations of DY-9760e or W-7. The IC_{50} values for DY-9760e and W-7 were 40 and 62 μM, respectively. The NOS activities were measured using 10 and 1 μM [^{3}H]arginine as substrate in panels A and B, respectively. Data are means of triplicate determinations from a representative experiment repeated twice with similar results. The standard errors were less than 5% in each experiment.

containing 10–15 μg protein in extracts of cerebellum and microglial cells was used to assess NOS activity. In the case of eNOS, which is localized in the microsomal fraction, endothelial cells were homogenized in 0.25 M sucrose solution containing 20 mM Tris–HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM DTT plus protease inhibitors. After the cell debris and nuclei were removed by a 10-min centrifugation at 1000 *g*, the microsomal fraction was obtained by 30 min of centrifugation at 10,000 *g*. The microsomal fractions were washed once with the above

solution and solubilized in 0.25 M sucrose containing 0.1% Triton X-100. An aliquot (10 μg protein) of the extracts was subjected to a NOS assay.

Assay of NOS Activities

NOS activity was measured by monitoring the conversion of [^{3}H]arginine to [^{3}H]citrulline as described [14]. The standard reaction mixture for the NOS assay contained 50 mM HEPES buffer, pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM NADPH, 10 μM FAD, 10 μM tetrahydrobiopterin (H_4B), 1 mg/mL of BSA, and 1 μM [^{3}H]arginine in a final volume of 100 μL. When eNOS or nNOS was assayed, 1 mM $CaCl_2$ and 1 μM CaM were included in the assay medium. After a 5-min incubation at 30°, the reaction was terminated with 1 mL of 20 mM HEPES buffer, pH 5.5, and 2 mM EDTA. After application to 0.5-mL columns of Dowex AG50WX-8 (Na^+ form), [^{3}H]citrulline was eluted with 1 mL water and quantified in a liquid scintillation counter. The test reagents were dissolved in DMSO and diluted with 50% DMSO. DMSO was included in the reaction mixture for NOS assays at a final concentration of 5%.

Assay of NO Production

Mouse neuroblastoma N1E-115 cells were cultured in DMEM (Nissui Pharmaceutical) supplemented with 10% FBS, 4 mM glutamine, 100 units/mL of potassium penicillin G, 100 μg/mL of streptomycin sulfate, and 25 ng/mL of fungizone (Gibco-BRL) at 37° in a 5% CO_2, 95% air humidified atmosphere. Differentiation of N1E-115 cells was accomplished by the method of Kimhi *et al.* [7]. Cells were plated at 2×10^5 cells/cm^2 in DMEM containing 10% FBS and 2% DMSO in 96-well culture plates and allowed to differentiate into mature neuronal cells for 2–3 days. After differentiation, cells were stimulated by treatment with 20 μM A23187 in the presence or absence of various concentrations of test drugs in FBS-free DMEM. Culture media were harvested 48 hr later and assayed for nitrite (NO_2^-) production by a spectrophotometric assay based on the Griess reaction [18].

Assay of CaM Kinase II Activity

Cultured hippocampal neurons were stimulated for 3 min by treatment with 10 μM glutamate in the presence or absence of various concentrations of DY-9760e. The cells were scraped from the plates and homogenized in solubilization buffer containing 0.1% Triton X-100, 50 mM HEPES, pH 7.5, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7$, 100 mM β-glycerophosphate, 25 mM NaF, 0.1 mM leupeptin, 50 μg/mL of trypsin inhibitor, 75 μM pepstatin A, 100 nM calyculin A, 1 mM sodium orthovanadate, and 1 mM DTT as reported [15]. The insoluble material was removed by centrifugation for 2 min at 15,000 *g* at 4°, and aliquots (5 μL) were used to determine protein content. The supernatants were treated with Laemmli's sample solution and boiled for 5 min at 100°. CaM kinase II activity was assessed by measurement of Ca^{2+}-independent activity using the in-gel kinase assay of Geahlen *et al.* [19] and Kameshita and Fujisawa [20] with some modifications as described [21]. Briefly, MBP was added as substrate to the SDS–polyacrylamide gel prior to polymerization of acrylamide. Cell lysates (15–20 μg protein) were separated by 10% SDS–PAGE. The gel then was treated for 1 hr with 6 M guanidine–HCl to denature the enzyme, followed by renaturation for 16 hr in 50 mM Tris–HCl buffer (pH 7.5) containing 0.04% Triton X-100, 5 mM mercaptoethanol, and 0.1 mM sodium orthovanadate at 4°. After renaturation, the gel was preincubated for 1 hr at 22° with 10 mL of 40 mM HEPES buffer (pH 8.0) containing 2 mM DTT, 10 mM $MgCl_2$, and 0.1 mM sodium orthovanadate. Phosphorylation of MBP was carried out by incubating the gel for 1 hr at 30° with 10 mL of 40 mM HEPES buffer (pH 8.0) containing 0.5 mM EGTA, 10 mM $MgCl_2$, 0.1 mM sodium orthovanadate, and 40 μM [γ-^{32}P]ATP (25 μCi). Then the gel was rinsed with 5% (w/v) trichloroacetic acid solution containing 1% sodium pyrophosphate to remove noncovalently bound ^{32}P. The gel was dried, and the amount of ^{32}P incorporation into MBP in the gel was quantified by a Bio-Imaging analyzer (BA100; Fujifilm) to determine CaM kinase II activity.

Data Analysis

K_i values were calculated by Dixon analysis. Statistically significant differences between treatment groups were determined by one-way ANOVA followed by Dunnett's test. *P* values less than 0.05 were considered significant.

RESULTS

Inhibition of Purified nNOS by DY-9760e and W-7

Since we have recently observed an inhibitory action of DY-9760e on various Ca^{2+}/CaM-dependent enzymes [5], its effect on purified nNOS activity was examined. The 150-kDa nNOS purified from rat cerebellum was homogeneous by SDS–PAGE, as shown in Fig. 1A, and the EC_{50} for CaM was 6 nM as reported previously [14]. In the presence of 10 nM CaM, the IC_{50} for DY-9760e was 40 μM, which was similar to that for W-7 (IC_{50} = 62 μM), a prototype CaM antagonist [22] (Fig. 1B). The K_i values of DY-9760e and W-7 for nNOS were 0.9 and 7.6 μM, respectively (Fig. 2). The K_i value for nNOS was comparable to that for CaM kinase II and CaM-dependent phosphodiesterase, but not to that for CaM kinase IV and myosin light chain kinase as reported [5]. Although the K_i value for the CaM-dependent protein phosphatase calcineurin was reported to be 2.0 μM using paranitrophenyl phosphate as substrate [5], the K_i value was much higher (K_i = 24 μM) when [^{32}P]casein was used as substrate in this study (data not shown).

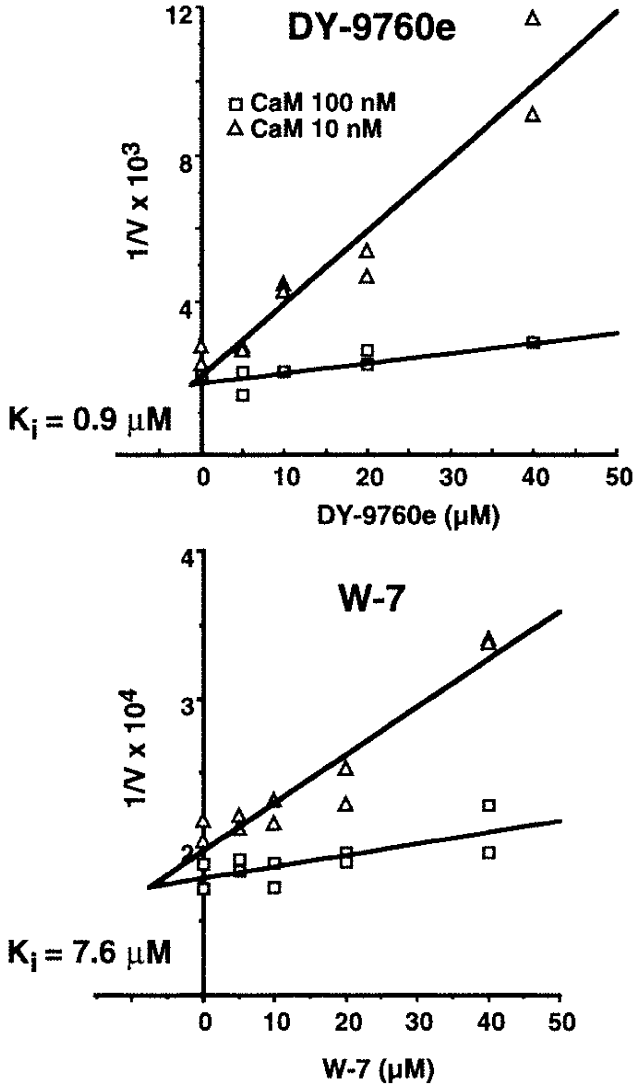

FIG. 2. K_i for DY-9760e and W-7 in inhibition of purified nNOS. In the presence of 10 or 100 nM CaM, NOS activity was assayed in the presence of various concentrations of DY-9760e or W-7. The K_i values were determined by a Dixon plot analysis.

Inhibition of nNOS, eNOS, and iNOS in Cell Extracts

We next tested the effects of DY-9760e on other NOS activities using crude preparations. Consistent with the results with purified nNOS, DY-9760e inhibited nNOS activity extracted from rat cerebellum in a concentration-dependent manner (Fig. 3). The IC_{50} of both DY-9760e and W-7 was about 200 μM. Similarly, eNOS extracted from HUVEC was also inhibited with the same potency as that seen with crude nNOS. In contrast, iNOS derived from LPS-stimulated microglial cells was insensitive to DY-9760e as well as to W-7. This is consistent with the observation that, although iNOS contains endogenous CaM in its own molecule, the enzyme activity is independent of Ca^{2+}/CaM. Because the cell extracts contained endogenous CaM, the IC_{50} values for crude nNOS and eNOS were much higher than that of purified nNOS, as shown in Fig. 1B.

Inhibition of nNOS Activity in N1E-115 Cells

Since DY-9760e has a potent inhibitory action on purified and crude CaM-dependent NOS, we examined further whether DY-9760e acts *in situ* on NOS. To address this question, we stimulated nNOS in neuroblastoma N1E-115

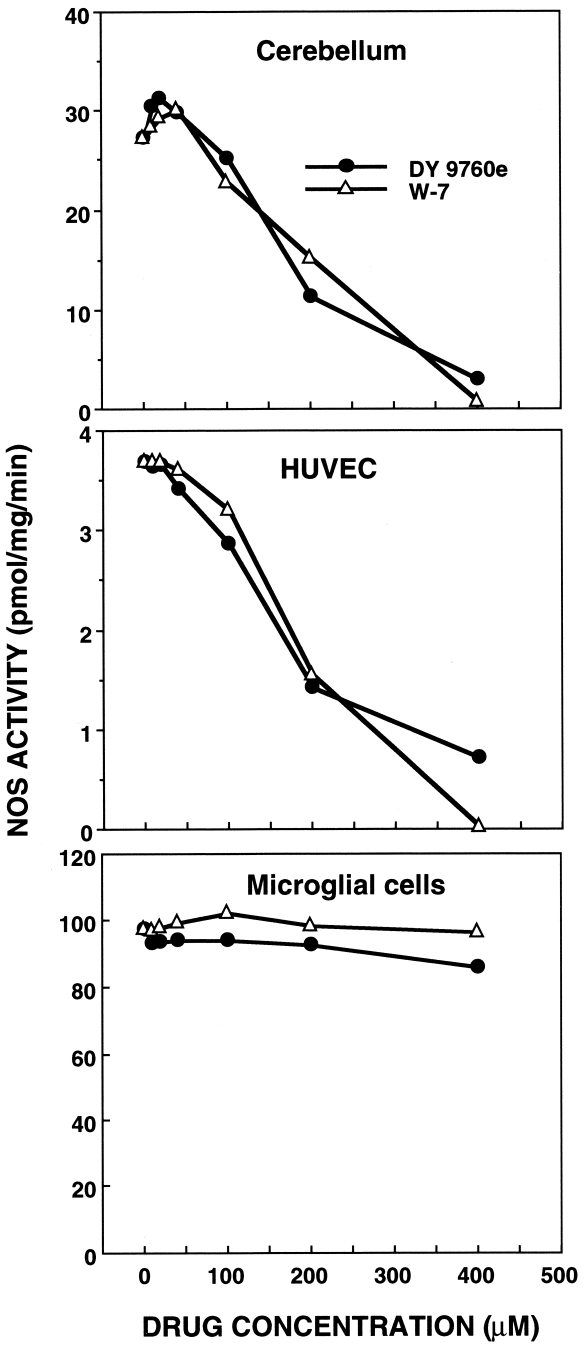

FIG. 3. Effects of DY-9760e and W-7 on crude nNOS, eNOS, and iNOS. Crude extracts were prepared from rat cerebellum, cultured HUVEC, and rat brain microglia as described in Materials and Methods. NOS activities in these extracts were assayed in the presence of 1 μM CaM and various concentrations of DY-9760e or W-7. Data are means of triplicate determinations from a representative experiment repeated twice with similar results. The standard errors were less than 5% in each experiment.

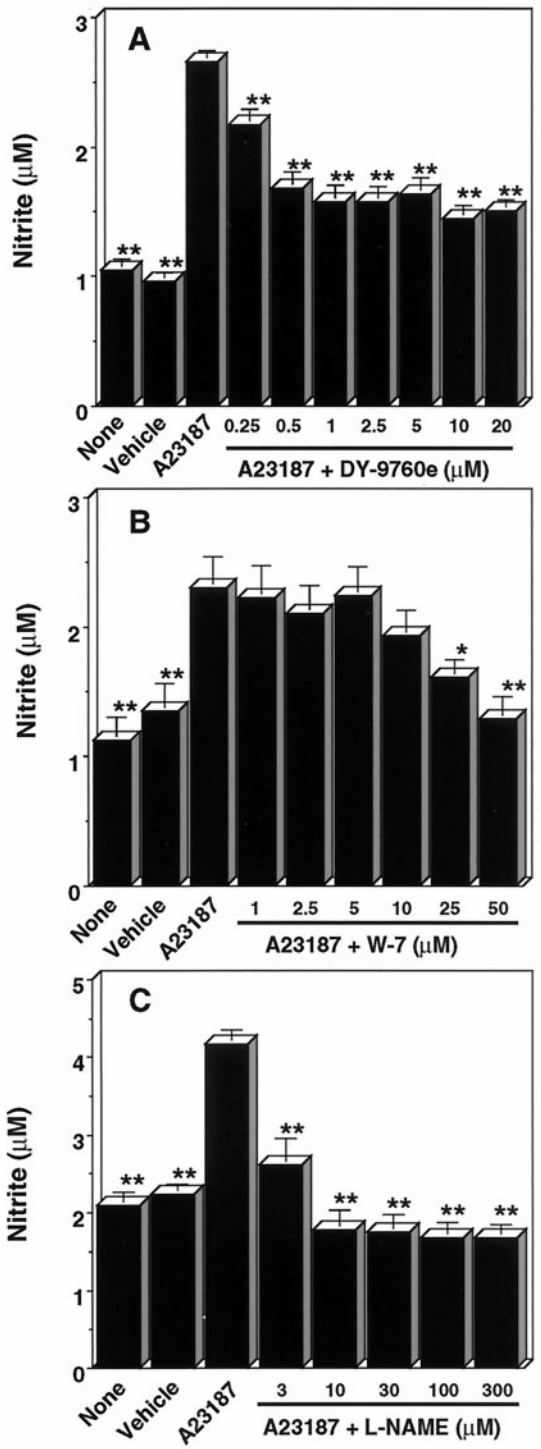

FIG. 4. Effects of DY-9760e, W-7, and L-NAME on A23187-induced NO production in cultured N1E-115 cells. N1E-115 cells were differentiated as described in Materials and Methods. After differentiation, cells were treated for 48 hr in 200 μL of the medium with vehicle (2% DMSO in FBS-free DMEM) or various concentrations of test drugs dissolved in 2% DMSO in FBS-free DMEM. Culture media (100 μL) were removed 48 hr later and assayed for nitrite (NO_2^-) production by a spectrophotometric assay based on the Griess reaction. Values are means ± SEM (N = 6). Values significantly different from that of A23187-stimulated cells are marked with an asterisk(s): (*) $P < 0.05$, and (**) $P < 0.01$.

cells by treatment with the calcium ionophore A23187, and measured the formation of nitrite, a metabolic product of NO. Prolonged incubation for 48 hr with A23187 increased the production of nitrite by 270%, compared with no treatment (none) or incubation with solvent alone (vehicle) as shown in Fig. 4A. DY-9760e significantly inhibited the production of NO at 0.25 μM and maximally at 0.5 μM (Fig. 4A). In contrast, 25 μM W-7 was required to inhibit A23187-induced NO production (Fig. 4B). To confirm that A23187-induced nitrite formation was due to activation of NOS in N1E-115 cells, we tested the effects of L-NAME, a potent inhibitor of NOS. A 10 μM concentration of L-NAME inhibited nitrite formation by A23187 completely without effect on the basal level of nitrite concentration (Fig. 4C).

Inhibition of CaM Kinase II in Cultured Hippocampal Neurons

Since DY-9760e is equally potent in inhibiting *in vitro* both purified nNOS and purified CaM kinase II, we next tested its effect on CaM kinase II *in situ*. Cultured rat hippocampal neurons were stimulated with 10 μM glutamate, and Ca^{2+}-independent activity was assessed by the in-gel kinase assay as reported [21]. Interestingly, 10 μM DY-9760e was required to inhibit the CaM kinase II activity in cultured hippocampal neurons (Fig. 5). Due to the insolubility of DY-9760e in the culture medium, we could not test

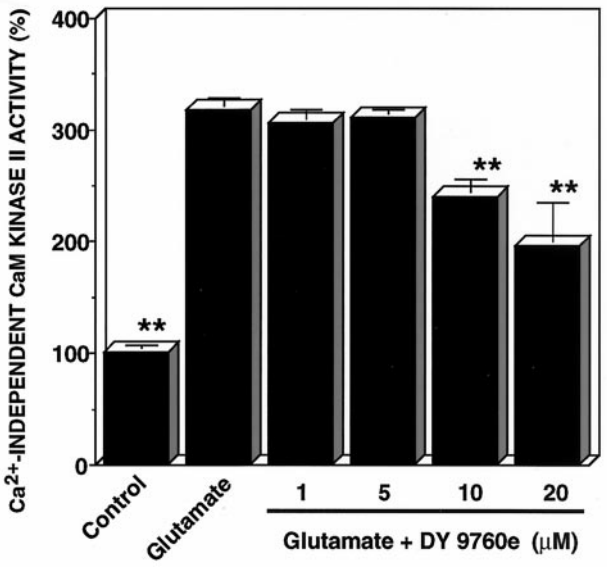

FIG. 5. Effects of DY-9760e on glutamate-induced CaM kinase II activation in cultured hippocampal neurons. Cultured cells were preincubated with Krebs–Ringer–HEPES for 30 min and incubated for 3 min with or without 10 μM glutamate in the presence or absence of various concentrations of DY-9760e. Activation of CaM kinase II was assayed by measurement of the Ca^{2+}-independent activity as described in Materials and Methods. Values are means ± SEM (N = 4) and are expressed as percent of the control values. Key: (**) indicates values significantly different ($P < 0.01$) from that of glutamate-stimulated cells.

concentrations higher than 20 μM. Together with the results of NOS production in N1E-115 cells, less than 5 μM DY-9760e could selectively inhibit CaM-dependent NOS activity *in situ*.

DISCUSSION

We previously reported that DY-9760e inhibits the various CaM-dependent enzymes with a competitive manner for CaM [5]. In addition, the drug has a neuroprotective effect in transient focal brain ischemia [6]. To analyze the molecular mechanisms underlying its neuroprotective action, we investigated the effects of DY-9760e on NOS activity *in vitro* and *in situ*. As expected, DY-9760e inhibited both nNOS and eNOS in crude preparations, and the K_i value (K_i = 0.9 μM) for purified nNOS was similar to that for purified CaM kinase II (K_i = 1.4 μM), as previously reported [5]. However, DY-9760e also had a potent inhibitory action *in situ* on nNOS activity in N1E-115 cells but only a modest inhibitory effect on CaM kinase II in cultured hippocampal neurons. For example, less than 5 μM DY-9760e strongly inhibited nNOS activity without affecting CaM kinase II activity. Consistent with its inhibitory effect on A23187-induced NO production, 1 μM DY-9760e significantly inhibited cell death induced by A23187 in N1E-115 cells [5]. Although the molecular mechanism underlying the potent inhibitory action *in situ* on nNOS activity is not clear, special localization and/or specific interaction *in situ* of DY-9760e with nNOS may account for its predominant effect on nNOS in intact cells.

Since glutamate-induced neurotoxicity and brain ischemia cause long-lasting inactivation of CaM kinase II in hippocampal neurons [23–25], we hypothesized that DY-9760e could have a protective effect on overactivation and subsequent inactivation of CaM kinase II during glutamate-induced neurotoxicity and brain ischemia. However, DY-9760e had no protective effect on the inactivation of CaM kinase II induced by high concentrations of glutamate in cultured hippocampal neurons (data not shown). In this context, DY-9760e has no significant effect on CaM kinase II *in situ*.

Although DY-9760e inhibits calcineurin *in vitro*, the K_i value (K_i = 24 μM) obtained with [^{32}P]casein as substrate was higher than that (K_i = 2.0 μM) obtained with paranitrophenyl phosphate as substrate. We investigated the effect of DY-9760e on calcineurin activity further in cultured hippocampal neurons by measurement of *in situ* phosphorylation of inhibitor 1. Inhibitor 1 is a known substrate for calcineurin and is dephosphorylated following stimulation of glutamate receptors in cultured hippocampal neurons (Fukunaga *et al.*, unpublished observation). We tested the effect of DY-9760e on glutamate-induced dephosphorylation of inhibitor 1. There was no significant effect on dephosphorylation up to 10 μM (data not shown). Therefore, we concluded that calcineurin was not a target enzyme for DY-9760e *in situ*.

Finally, we determined whether CaM-dependent phosphodiesterase is inhibited by DY-9760e *in situ* by investigating the effects of DY-9760e on cyclic AMP levels in cultured hippocampal neurons before and after stimulation with glutamate. As previously reported [26], cyclic AMP accumulated following stimulation with glutamate. DY-9760e, however, had no significant effect on either basal or glutamate-stimulated cyclic AMP accumulation (data not shown). This finding suggests that DY-9760e does not interfere with CaM-dependent phosphodiesterase. Taken together with these results, DY-9760e appears to be a selective inhibitor for CaM-dependent NOS *in situ*, and its inhibitory effect on NOS can account for the neuroprotective effect during brain ischemia.

Increasing evidence suggests that NO is involved in cell death during glutamate-induced neurotoxicity in cultured neurons and brain ischemia. Indeed, NOS inhibitors block neuronal injury [1, 27, 28]. Consistent with these observations, DY-9760e has a potent neuroprotective effect in transient focal cerebral ischemia [6]. Suppression of production of NO and/or the toxic oxidant peroxynitrite ($ONOO^-$) possibly accounts for the neuroprotective action of DY-9760e during brain damage. However, other targets of DY-9760e may also be involved in its neuroprotective action. CaM has been implicated in the homeostasis of intracellular Ca^{2+} concentrations in the cytosol through regulation of voltage-dependent calcium channels [29] and calcium release channels such as the inositol-1,4,5-phosphate receptor [30–32] and the ryanodine receptor [33]. The effect of DY-9760e on intracellular Ca^{2+} concentrations during neurotoxic conditions is now under investigation.

A major aim of the present study was to investigate the molecular mechanisms underlying the neuroprotective action of DY-9760e, a new inhibitor for CaM. These *in vitro* and *in situ* studies demonstrated that CaM-dependent NOS is an important target molecule for the drug, and the inhibitory effects on NOS possibly account for its neuroprotective effect. Our results also suggest that a CaM antagonist is a new therapeutic agent capable of protecting neurons from damage. Further investigation is required to address how DY-9760e selectively interacts with CaM-dependent NOS *in situ* and whether the inhibitory effects on both nNOS and eNOS account for the neuroprotective action of DY-9760e in transient focal cerebral ischemia.

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