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DY-9760e, a novel calmodulin antagonist with cytoprotective action

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Abstract

We report the pharmacological characterization and cytoprotective effect of DY-9760e, 3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-indazole dihydrochloride 3.5 hydrate, a novel antagonist of calmodulin. DY-9760e inhibited calmodulin-dependent enzymes, including calmodulin-dependent phosphodiesterase and myosin light chain kinase with K_i values of 1.4, 12, 2.0, 3.8 and 133 μM, respectively. These antagonistic effects of DY-9760e were more potent than those of W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, another calmodulin antagonist. This compound showed little or no effect on calmodulin-independent enzymes, such as protein kinase A and C and calpain I and II. Analysis of the hydrophobic interaction of DY-9760e with calmodulin by using 2-*p*-toluidinylnaphthalene-6-sulfonate and 9-anthroylcholine revealed that, like W-7, DY-9760e bound to the hydrophobic regions of calmodulin. The [^{14}C]DY-9760e binding assay indicated that DY-9760e bound to calmodulin at one class of binding site. Finally, DY-9760e substantially protected N1E-115 neuroblastoma cells from cytotoxicity induced by the Ca^{2+} ionophore, A23187. These results indicate that DY-9760e, a novel calmodulin antagonist, possesses a cytoprotective action and suggest that calmodulin plays a critical role in mediating some of the biochemical events leading to cell death following Ca^{2+} overload. © 1997 Elsevier Science B.V.

Keywords: DY-9760e; W-7; Calmodulin antagonist; Cytotoxicity; Neuroprotection

1. Introduction

Ca^{2+} overload is thought to play an important role in the pathological events following brain ischemia (Choi, 1988; Meldrum and Garthwaite, 1990; Mitani et al., 1993; Choi, 1995). During cerebral ischemia, elevated levels of extracellular glutamate cause excessive stimulation of its postsynaptic receptors, including the NMDA receptor and subsequently induce Ca^{2+} influx into neurons (Choi, 1988; Meldrum and Garthwaite, 1990; Takagi et al., 1993). Elevation of the Ca^{2+} concentration in neurons disrupts the ionic balance and activates various Ca^{2+}-dependent enzymes and Ca^{2+}-binding proteins.

Calmodulin is a major Ca^{2+}-binding protein found in the central nervous system (Zhou et al., 1985; James et al., 1995). Calmodulin is implicated in a variety of cell functions through the activation of calmodulin-dependent enzymes, such as phosphodiesterase, protein kinases, protein phosphatase and nitric oxide (NO) synthase (James et al., 1995). Thus, Ca^{2+} overload in neurons induced by ischemic insult may overactivate Ca^{2+}/calmodulin-dependent pathway(s) and lead to irreversible cell damage. In fact, immunohistochemical studies have shown that persistent Ca^{2+}/calmodulin-binding in neurons is associated with ischemic neuronal damage (Picone et al., 1989).

The role of calmodulin in a variety of systems has been studied by using calmodulin antagonists, including naphthalenesulfonamides such as W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, phenothiazines such as trifluoperazine and imidazolium compounds such as calmidazolium. The calmodulin antagonists, W-7 and calmidazolium, protect cultured neurons against NMDA-induced neuronal cell death (Dawson et al., 1993b). Furthermore, some inhibitors of calmodulin-dependent enzymes, including calcineurin (Dawson et al., 1993a; Sharkey and Butcher, 1994; Ide et al., 1996), calmodulin-dependent protein kinase II (Hajimohammadreza et al., 1995) and NO synthase (Nagafuji et al., 1992; Buisson et al., 1993; Dawson et al.,

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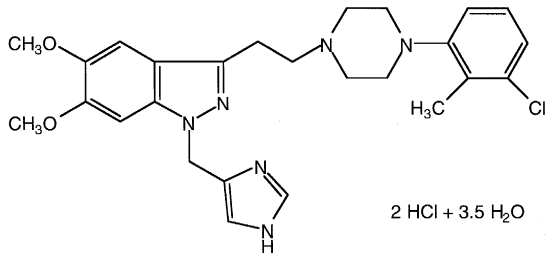

Fig. 1. Chemical structure of DY-9760e.

1993b), provide neuroprotective effects against NMDA-induced cell death or ischemic brain damage. These findings suggest the important role of Ca^{2+}/calmodulin-dependent pathway(s) in mediating some of the biochemical events leading to cell death following Ca^{2+} overload.

In the present study, we describe how a potent and novel class of calmodulin antagonist, DY-9760e, 3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-indazole dihydrochloride 3.5 hydrate, an indazole derivative (Fig. 1), has a cytoprotective effect against cell death induced by a Ca^{2+} ionophore in neuroblastoma cells.

2. Materials and methods

2.1. Reagents

DY-9760e was synthesized at Daiichi Pharmaceutical (Tokyo, Japan). [^{14}C]DY-9760e (54.1 mCi/mmol) was obtained from Daiichi Pure Chemical (Tokyo, Japan). W-7 and H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, were purchased from Seikagaku (Tokyo, Japan). Calmodulin (bovine brain), calcineurin (bovine brain), calmodulin-dependent phosphodiesterase (bovine brain), cyclic AMP-dependent protein kinase (protein kinase A, catalytic subunit, bovine heart), calpain II (rabbit skeletal muscle), *p*-nitrophenyl phosphate, 9-anthroylcholine, 2-*p*-toluidinylnaphthylene-6-sulfonate (TNS), A23187 and bovine serum albumin were obtained from Sigma (St. Louis, MO, USA). Protein kinase C (a mixture of α, β and γ isoforms, bovine brain) was from Upstate Biotechnology (Lake Placid, NY, USA). Calpain I (porcine erythrocyte), calpain inhibitor I and II and staurosporine were purchased from Calbiochem (La Jolla, CA, USA). Calmodulin-dependent protein kinase II (Fukunaga et al., 1982) and calmodulin-dependent protein kinase IV (Miyano et al., 1992) were purified from rat brain as described previously. Myosin light chain kinase and myosin light chain were purified from chicken gizzard according to the method of Walsh et al. (1983) and Miyamoto et al. (1981), respectively. [^{3}H]Cyclic GMP and [^{32}P]ATP were from Dupont-New England Nuclear (Boston, MA, USA). Test compounds were dissolved in dimethyl sulfoxide and diluted in reaction mixtures or culture medium. The final concentrations of dimethyl sulfoxide were 1–5% for the cell-free assay and 2.5% for the cytotoxicity assay.

2.2. Assay of calcineurin activity

Calcineurin activity was assayed by the method of Pallen and Wang (1984) with some modifications. The reaction mixture contained 50 mM Tris–HCl (pH 7.2), 2.91 μg/ml calcineurin, 25 mM *p*-nitrophenyl phosphate, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $NiCl_2$, 0.25 mg/ml bovine serum albumin, 215–858 ng/ml calmodulin and test compounds in a volume of 200 μl. The reactions were initiated by the addition of calmodulin and *p*-nitrophenyl phosphate and changes in absorbance at 405 nm were continuously monitored. Calmodulin-dependent activity was estimated by subtracting activity in the absence of calmodulin from that in the presence of calmodulin.

2.3. Assay of calmodulin-dependent phosphodiesterase activity

Phosphodiesterase activity was measured according to the method of Thompson and Appleman (1971) with some modifications. The reaction mixture contained 50 mM Tris–HCl (pH 7.5), 0.67 μg/ml calmodulin-dependent phosphodiesterase, 17.2–85.8 ng/ml calmodulin, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ or 1 mM EGTA, 0.1 mg/ml bovine serum albumin, 25 μM [^{3}H]cyclic GMP (10 000 dpm/nmol) and test compounds in a volume of 500 μl. After incubation at 30°C for 10 min, the reactions were terminated by boiling. [^{3}H]GMP produced by phosphodiesterase was broken down to [^{3}H]guanosine by snake venom (phosphodiesterase I type V from *Bothrops atrox*, Sigma) and the remaining [^{3}H]cyclic GMP was absorbed by anion exchange resin (AG1-X8, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA, USA). [^{3}H]Guanosine in the supernatant was counted with a liquid scintillation counter.

2.4. Assay of calmodulin-dependent protein kinase II and IV activities

Calmodulin-dependent protein kinase II and IV activities were measured as described previously (Fukunaga et al., 1989). The standard kinase assay contained 50 mM HEPES buffer (pH 7.5), 10 mM magnesium acetate, 0.1 mM $CaCl_2$ or 1 mM EGTA, 1 mg/ml bovine serum albumin, 40 μM syntide-2 (Bachem, Bubendorf, Switzerland), 0.1 mM [γ-^{32}P]ATP (3000–5000 cpm/pmol), calmodulin, purified enzyme and test compounds in a final volume of 25 μl. The concentrations of calmodulin and enzymes in each assay were as follows: 85.8–343 ng/ml and 0.4 μg/ml for calmodulin-dependent protein kinase II and 0.858–1.72 μg/ml and 6 μg/ml for calmodulin-dependent protein kinase IV. The reactions were carried out at 30°C for 4 min. Incorporation of ^{32}P into syntide-2 was measured according to the method of Roskoski (1983). After incubation, 15 μl aliquots were spotted on phosphocellulose paper squares. The squares were washed with 75 mM phosphoric acid and the radioactivity was quantified.

2.5. Assay of myosin light chain kinase activity

Myosin light chain kinase activity was measured as reported previously (Walsh et al., 1983) in a reaction mixture containing 50 mM HEPES buffer (pH 7.5), 10 mM magnesium acetate, 0.1 mM $CaCl_2$ or 1 mM EGTA, 1 mg/ml bovine serum albumin, 10 μM myosin light chain, 0.1 mM [γ-^{32}P]ATP (3000–5000 cpm/pmol), 4.29–17.2 ng/ml calmodulin, 0.6 μg/ml myosin light chain kinase and test compounds in a volume of 25 μl. The reactions were initiated by the addition of [γ-^{32}P]ATP. After a 4 min incubation at 30°C, 15 μl aliquots were spotted on Whatman 3 MM paper squares and the papers were washed with 5% trichloroacetic acid solution.

2.6. Assay of protein kinase A and protein kinase C activity

Phosphorylation activity of protein kinase A was assayed with the non-radioisotopic protein kinase assay kit (Medical and Biological Laboratories, Nagoya, Japan) by using 1.28 μg/ml of protein kinase A subunit. The activity of protein kinase C was measured using the protein kinase C enzyme assay system (Amersham, Amersham, UK) by using 0.1 μg/ml protein kinase C.

2.7. Assay of calpain I and II activity

Ca^{2+}-dependent azocaseinolytic activity was measured by the method of Moss et al. (1991) with some modifications. The reaction mixture contained 50 mM Tris–HCl (pH 7.4), 15 mM 2-mercaptoethanol, 2 mg/ml azocasein, $CaCl_2$, purified enzyme and test compounds in a volume of 500 μl. The concentrations of purified enzymes and $CaCl_2$ were as follows: 15 μg/ml and 50 μM for calpain I and 60 μg/ml and 5 mM for calpain II, respectively. Ca^{2+}-independent activity was measured in the presence of 2 mM EGTA. The reactions were performed at 25°C for 2 h and terminated by the addition of 15% trichloroacetic acid. The reaction mixture was placed in a freezer (−17°C) and centrifuged. 0.5 M NaOH was added to the supernatant and the absorbance of the solution was measured at 440 nm.

2.8. Fluorescence measurements

Two hydrophobic probes, TNS and 9-anthroylcholine, were used. Fluorescence was measured at room temperature as reported by Epstein et al. (1982) with some modifications. The reaction mixture contained 50 mM Tris–HCl (pH 7.5), 14.9 μg/ml calmodulin, 1 mM $CaCl_2$ or 0.3 mM EGTA, 20 μM probe (TNS or 9-anthroylcholine) and test compounds. Excitation was at 365 nm for both probes and emission intensity was measured from 380–580 nm. The test compounds were added to the reaction mixture cumulatively.

2.9. [^{14}C]DY-9760e binding to calmodulin

The binding of [^{14}C]DY-9760e to calmodulin was assayed by the equilibrium gel-diffusion method (Hirose and Kano, 1971). Binding studies were performed in a reaction mixture containing 20 mM Tris–HCl buffer (pH 7.5), 20 mM imidazole, 3 mM $MgCl_2$, 0.125 mg/ml bovine serum albumin, 0.1 mM $CaCl_2$, 0.1 mg/ml calmodulin and [^{14}C]DY-9760e (2–40 μM) in the presence of 50 mg of dried Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden). The mixture was incubated at 25°C for 1 h. The solution outside the gel was withdrawn and the radioactivity was measured. Nonspecific binding was defined by the inclusion of 2 mM EGTA.

2.10. Ca^{2+}-induced cell death

Mouse N1E-115 neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (Dainippon Pharmaceutical, Osaka, Japan), 4 mM glutamine, 100 units/ml potassium penicillin G, 100 μg/ml streptomycin sulfate and 250 ng/ml fungizone (Gibco-BRL, Gaithersburg, MD, USA) at 37°C in 5% CO_2, 95% air-humidified atmosphere. Differentiation of N1E-115 cells was accomplished by the method of Kimhi et al. (1976). Cells were plated at approximately 2×10^5 cells/cm^2 in DMEM containing 10% fetal calf serum and 2% dimethylsulfoxide in 48-well culture plates and were allowed to differentiate into mature nerve cells for 6–7 days. The medium containing dimethyl sulfoxide was changed every 2–3 days. Cell death was induced by exposing the cells to A23187 (10 μM). Test compounds were applied to the cells 1 h before the A23187 treatment. Quantitative assessment of cell injury was done by measuring lactate dehydrogenase (LDH) release as a marker for membrane breakage and cell death (Koh and Choi, 1987). LDH levels in the culture medium 24 h after A23187 exposure were measured with the MTX 'LDH' kit (KYOKUTO Pharmaceutical, Tokyo, Japan). The extent of cell death was expressed as a percentage of total LDH activity in cultured cells after treatment with 0.1% Triton X-100.

2.11. Data analysis

Analysis of data to determine IC_{50}, K_d and r_{max} (maximum moles of [^{14}C]DY-9760e bound per mol of calmodulin) values was performed using standard equations. K_i values were obtained from Dixon analysis. Statistically significant differences between treatment groups were determined by one-way analysis of variance followed by Dunnett's test. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Inhibition of calmodulin-dependent enzymes by DY-9760e and W-7

The effects of DY-9760e on calmodulin-dependent enzymes, including calcineurin, calmodulin-dependent phosphodiesterase, calmodulin-dependent protein kinase II and IV and myosin light chain kinase, were determined and compared with those of W-7. Fig. 2 shows that DY-9760e and W-7 inhibited the activity of calmodulin-activated calcineurin in a concentration-dependent manner. Further mutual interaction was investigated by measuring calcineurin activity at various concentrations of calmodulin in the presence of these antagonists. The kinetic analysis revealed that the inhibitory action of these antagonists was competitive with respect to calmodulin (Fig. 3). K_i values of DY-9760e and W-7 were estimated to be 2.0 and 137 μM, respectively. In addition, DY-9760e and W-7 did not affect calmodulin-independent calcineurin activity.

K_i values of DY-9760e and W-7 for the other calmodulin-dependent enzymes are presented in Table 1. The K_i values of DY-9760e for calmodulin-dependent phosphodiesterase, calmodulin-dependent protein kinase II and IV and myosin light chain kinase were 3.8, 1.4, 12 and 133 μM, respectively. DY-9760e was most potent in its inhibition of calmodulin-dependent protein kinase II, whereas a higher concentration was needed to inhibit myosin light chain kinase. DY-9760e was 2–70 times more potent than W-7 in inhibiting calmodulin-dependent enzymes.

3.2. Effects of DY-9760e on calmodulin-independent enzymes

The effects of DY-9760e, W-7 and related agents on protein kinase A, protein kinase C and calpain I and II

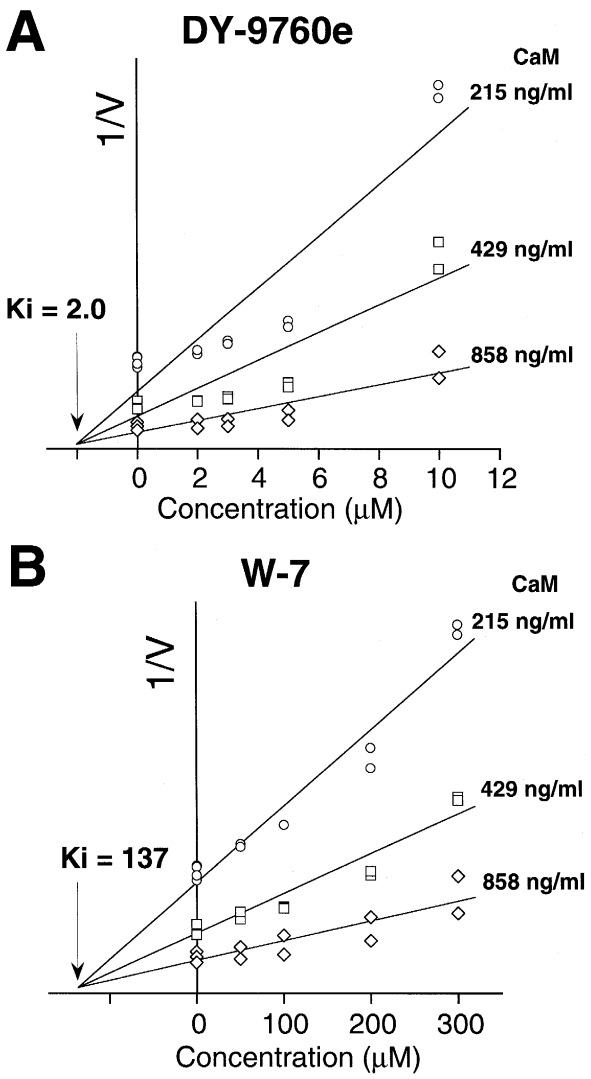

Fig. 3. Kinetic analysis of inhibition by DY-9760e (A) and W-7 (B) of calcineurin activity. Kinetic analysis was done by using a Dixon plot. Calcineurin activity was measured in the presence of 1 mM $CaCl_2$ and various concentrations of calmodulin (215–858 ng/ml) and of the test compound. Values are data for each sample in one experiment.

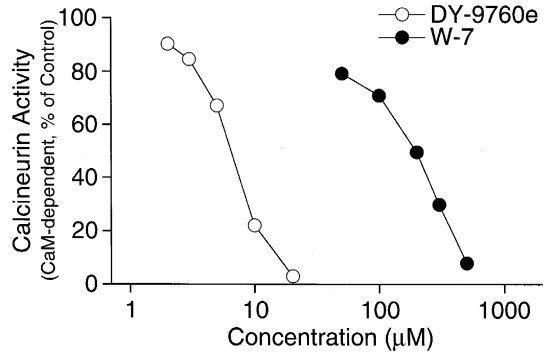

Fig. 2. Effects of DY-9760e and W-7 on calcineurin activity. Calcineurin activity was assayed as described in Section 2. The concentrations of calmodulin and calcineurin were 429 ng/ml and 2.91 μg/ml, respectively. Values are means of triplicate samples in one experiment and are plotted as percent inhibition of enzyme activity versus the concentrations of calmodulin antagonists.

Table 1
Inhibition of calmodulin-dependent enzymes by DY-9760e and W-7

Enzymes	K_i (μM)	
	DY-9760e	W-7
CaM kinase II	1.4	10
Calcineurin	2.0	137
PDE	3.8	8.1
CaM kinase IV	12	35
MLCK	133	90

Assays of calmodulin-dependent enzymes were performed as described in Section 2. K_i values were obtained from Dixon analysis. Abbreviations: CaM kinase II, calmodulin-dependent protein kinase II; PDE, calmodulin-dependent phosphodiesterase; CaM kinase IV, calmodulin-dependent protein kinase IV; MLCK, myosin light chain kinase.

Table 2
Actions of DY-9760e and W-7 on calmoulin-independent enzymes

Enzymes	IC_{50} (μM)	
	DY-9760e	W-7
Protein kinase A	91	> 400
Protein kinase C	128	301
Calpain I	> 300	> 300
Calpain II	> 100	ND

Assays of calmodulin-independent enzymes were performed as described in Section 2. Results are expressed as IC_{50} values. Data are means of duplicate or triplicate measurements. ND, not determined.

were determined. DY-9760e inhibited protein kinase A and protein kinase C activity at 65–90 times higher concentrations than those for calmodulin-dependent protein kinase II (Table 2). W-7 inhibited protein kinase A and protein kinase C at 400 μM by 48% and 44%, respectively. DY-9760e and W-7 slightly inhibited calpain I at a high concentration (200–300 μM) by 25%. DY-9760e did not affect calpain II activities at concentrations up to 100 μM. IC_{50} values for reference inhibitors of each enzyme were as follows: staurosporine, 3 nM for protein kinase C and 30 nM for protein kinase A; H-7, 28 μM for protein kinase C; calpain inhibitor I, 100–300 nM for calpain I; calpain inhibitor II, approximately 300 nM for calpain II. These values were consistent with those of earlier studies (Hidaka et al., 1984; Sasaki et al., 1990; Takahashi et al., 1990; Sarin et al., 1993).

3.3. Interaction of DY-9760e with calmodulin

To test whether DY-9760e binds directly to the Ca^{2+}/calmodulin complex, we examined the effects of DY-9760e on the binding of the hydrophobic fluorescent probes, 9-anthroylcholine and TNS, to calmodulin. The intensity of the fluorescence of 9-anthroylcholine (Fig. 4A) and TNS (data not shown) was greatly increased by the addition of Ca^{2+} and calmodulin. DY-9760e suppressed the increase in fluorescence of these probes in a concentration-dependent manner (Fig. 4B,C). IC_{50} values of DY-9760e were 3.1 and > 30 μM for 9-anthroylcholine and TNS, respectively. W-7 also inhibited the fluorescence of 9-anthroylcholine and TNS with IC_{50} values of > 30 μM for 9-anthroylcholine and 8.1 μM for TNS, respectively (Fig. 4B and C).

The interaction of DY-9760e with calmodulin was further studied by using [^{14}C] DY-9760e and the equilibrium gel-diffusion method (Hirose and Kano, 1971). The binding of [^{14}C]DY-9760e to calmodulin increased in the presence of 0.1 mM $CaCl_2$, when compared with that in the presence of 2 mM EGTA (Fig. 5A). When the specific binding of [^{14}C]DY-9760e was analyzed by using the Scatchard equation, one type of [^{14}C]DY-9760e binding site was present with a K_d value of 16.3 ± 2.5 μM and an r_{max} value of 2.2 ± 0.2 mol/mol of calmodulin (mean standard error from four independent experiments) (Fig. 5B).

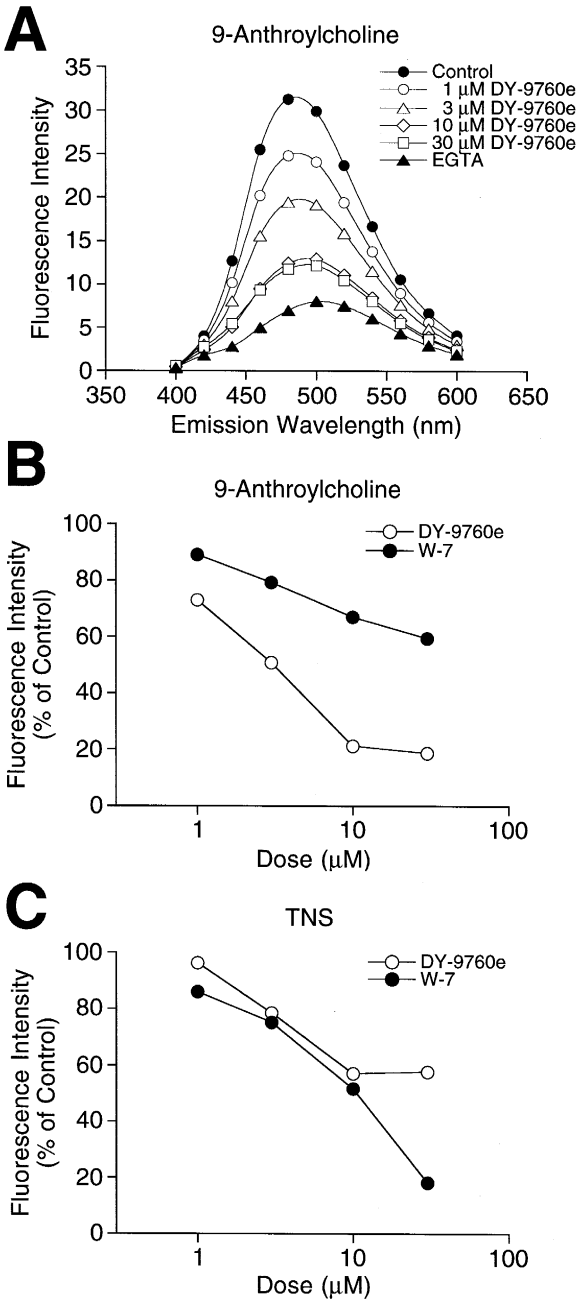

Fig. 4. Effects of DY-9760e and W-7 on the fluorescence intensity of the hydrophobic probes, 9-anthroylcholine and TNS. (A) Typical fluorescence of 9-anthroylcholine measured with or without DY-9760e. (B and C) Inhibitory effects of DY-9760e and W-7 on the fluorescence intensity of the probes. Emission intensity was measured at 480 nm for 9-anthroylcholine and at 460 nm for TNS, respectively, and expressed as percentages of that in the absence of the compounds.

3.4. Protective effects of DY-9760e and W-7 against Ca^{2+} ionophore-induced cell death in N1E-115 neuroblastoma cells

The cytoprotective effects of calmodulin antagonists against Ca^{2+} ionophore-induced cell injury were investigated in a neuroblastoma cell line, N1E-115, which is well-established in the study of neuronal differentiation (Kimhi et al., 1976). The cell injury was assessed by measurement of LDH activity released into the cultured medium. Exposure to A23187 (10 μM) for 24 h increased LDH release from the cells. DY-9760e significantly increased A23187-induced LDH release at concentrations of 1–10 μM (Fig. 6A), in a dose-dependent manner. W-7 also blocked Ca^{2+}-induced cytotoxicity, but concentrations at least 10-times higher than those of DY-9760e were needed to elicit a significant cytoprotective effect (Fig. 6B). DY-9760e and W-7 did not affect the basal level of LDH release in N1E-115 cells (data not shown).

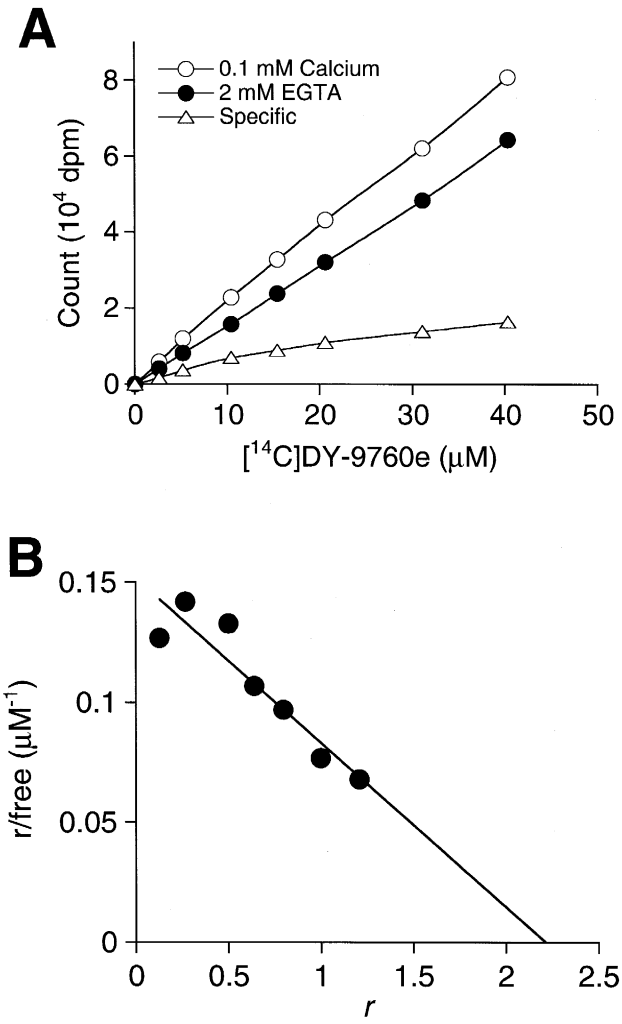

Fig. 5. Binding isotherms (A) and Scatchard plot (B) of [^{14}C]DY-9760e binding to calmodulin. Specific binding was defined as binding in the presence of 0.1 mM $CaCl_2$ minus binding in the presence of 2 mM EGTA. r: moles of [^{14}C]DY-9760e bound per mole of calmodulin. Each point represents the mean of duplicate determinations obtained in a representative experiment, which was repeated 4 times with comparable results.

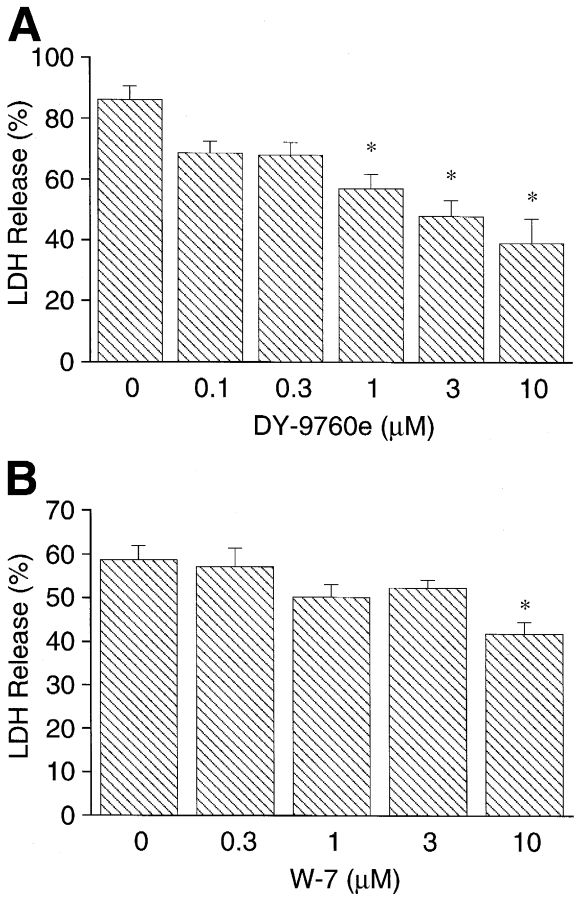

Fig. 6. Protective effects of DY-9760e and W-7 against A23187-induced cell death. N1E-115 neuroblastoma cells were treated with 10 μM A23187 for 24 h. Cell death was assessed by the measurement of LDH released into the cultured medium. Cells were treated with DY-9760e or W-7 for 1 h before A23187-exposure. Data are means ± standard error of six wells in a representative experiment. * $P < 0.01$ versus A23187 alone.

4. Discussion

The present study indicates that DY-9760e is a novel and structurally diverse antagonist of calmodulin which can attenuate cell injury more effectively than W-7, a typical and moderately potent calmodulin antagonist. DY-9760e inhibited calcineurin, calmodulin-dependent phosphodiesterase, calmodulin-dependent protein kinase II and calmodulin-dependent protein kinase IV with K_i values in the range of 1.4–12 μM. The inhibitory effects of DY-9760e on these calmodulin-dependent enzymes were 2–70 times more potent than those of W-7. In contrast, DY-9760e slightly inhibited myosin light chain kinase activity at higher concentrations ($K_i = 133$ μM), suggesting that it

has a weak effect on the vascular smooth muscle contraction system. Similarly, the slight inhibitory effect of this compound on protein kinase A, protein kinase C and calpain I and II indicates that the cytoprotective effect of DY-9760e is most likely exerted through the inhibition of calmodulin-dependent enzymes. DY-9760e inhibited the calmodulin-dependent enzymes by competing with calmodulin. Scatchard analysis indicated that [^{14}C]DY-9760e can bind to calmodulin at one class of site with a K_d value of 16 μM in the presence of Ca^{2+}. W-7 is known to interact with two classes of binding sites in calmodulin (Hidaka et al., 1980): a high-affinity site (K_d = 11 μM and numbers = 3) and a low-affinity site (K_d = 200 μM and numbers = 9). Thus the binding sites of DY-9760e on calmodulin may be different from those of W-7. This is consistent with the different inhibitory properties of DY-9760e and W-7 on TNS- and 9-anthroylcholine-induced fluorescence. Furthermore, DY-9760e and W-7 showed different selectivity for calmodulin-dependent enzymes: DY-9760e inhibited enzyme activity with the following order of potency, calmodulin-dependent protein kinase II > calcineurin > calmodulin-dependent phosphodiesterase > calmodulin-dependent protein kinase IV > myosin light chain kinase, whereas that of W-7 was calmodulin-dependent phosphodiesterase > calmodulin-dependent protein kinase II > calmodulin-kinase, calmodulin-dependent protein kinase IV > myosin light chain kinase > calcineurin. The selectivity of these calmodulin antagonists for calmodulin-dependent enzymes may be dependent on the location of the binding sites on calmodulin. Cook et al. (1994) reported that trifluoperazine interacts with residues in the C-terminal domain of calmodulin, as assessed by determination of the X-ray structure of the crystallized calmodulin/trifluoperazine complex. To better understand the calmodulin/DY-9760e complex, NMR and X-ray investigations are in progress.

Dawson et al. (1993b) have indicated that W-7 and calmidazolium prevent NMDA-induced neuronal cell death. An early biochemical event of NMDA-induced excitotoxicity in the neuron is disturbance of the ionic balance, especially the Ca^{2+} equilibrium. Ca^{2+} overload in neurons may elicit the activation/overactivation of Ca^{2+}-dependent enzymes such as calmodulin-dependent enzymes and calpains. Since the cytoprotective effect of DY-9760e was apparent at concentrations of 1–10 μM, calcineurin, calmodulin-dependent protein kinase II and calmomodulin-dependent phosphodiesterase may be involved in the protective effect of the compound. In the case of W-7, the cytoprotective effect may be due to the inhibition of calmodulin-dependent protein kinase II and calmodulin-dependent phosphodiesterase, but not calcineurin.

A relatively specific inhibitor of calmodulin-dependent protein kinase II, KN-62, is known to have a neuroprotective effect against NMDA- and hypoxia/hypoglycemia-induced neuronal cell death (Hajimohammadreza et al., 1995). Furthermore, glutamate-induced neurotoxicity and brain ischemia cause long-lasting inactivation of calmodulin-dependent protein kinase II and its translocation to the particulate fraction in cultured hippocampal neurons (Morioka et al., 1992, 1995). DY-9760e and W-7 may have a protective effect on the overactivation and then inactivation of calmodulin-dependent protein kinase II in glutamate-induced neurotoxicity as well as in brain ischemia. The cAMP-dependent pathway is also known to be involved in neuroprotective effects following metabotropic glutamate receptor stimulation during anoxia and NO toxicity (Nicoletti et al., 1995). Inhibition of calmodulin-dependent phosphodiesterase, which is abundant in neurons, by calmodulin antagonists may cause an accumulation of cAMP and in turn provide a neuroprotective effect.

There is another possible explanation for the cytoprotective effect of DY-9760e. It has been reported that FK506, an inhibitor of calneurin, suppresses glutamate-induced cell death and/or neuronal injury in experimental models of stroke (Dawson et al., 1993a; Sharkey and Butcher, 1994; Ide et al., 1996). Furthermore, calcineurin is shown to be a direct activator of apoptosis not accompanied by de novo protein synthesis in T-cells (Shibazaki and McKeon, 1995). At concentrations around the K_i values for the anti-calmodulin effects, DY-9760e (3 μM) and W-7 (10 μM) inhibited LDH release by 50% and 30%, respectively. These results suggest that mechanisms other than inhibition of calmodulin-dependent protein kinase II and calmodulin-dependent phosphodiesterase, probably an anti-calcineurin effect, are involved in the cytoprotective effect of DY-9760e. Finally, another attractive candidate for the target enzymes of DY-9760e is NO synthase. Indeed, NO is involved in cell death in cultures and in brain ischemic damage and NO synthase inhibitors ameliorate neuronal injury (Nagafuji et al., 1992; Buisson et al., 1993; Dawson et al., 1993b).

In conclusion, we investigated DY-9760e, a potent and novel class of calmodulin antagonist with a cytoprotective action. The findings of this study suggest that calmodulin plays a pivotal role in the processes of Ca^{2+}-induced cytotoxicity and that inhibition of calmodulin can attenuate neuronal injury. Further investigation is needed to evaluate the neuroprotective effects of DY-9760e in experimental models of brain ischemia and neurodegenerative diseases.

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