Protective Effect of DY-9760e, a Calmodulin Antagonist, against Neuronal Cell Death

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An excessive elevation of intracellular Ca$^{2+}$ levels is known to play a key role in the pathological events following cerebral ischemia. DY-9760e, 3-[[4-((3-chloro-2-methylphenylmethyl)-1-piperazinyl)ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-indazole dihydrochloride 3.5 hydrate, is a potent calmodulin antagonist that attenuates brain damage in focal ischemia models. In the present study, we investigated the effect of DY-9760e on neuronal cell death induced by a variety of cell-toxic stimuli that increase intracellular Ca$^{2+}$. Cell death was induced by the exposure of primary cultured neurons to excitotoxic agents such as glutamate and N-methyl-D-aspartate, membrane-depolarizing agents such as veratridine and high KCl, or thapsigargin an endoplasmic reticulum Ca$^{2+}$-ATPase inhibitor. Treatment with DY-9760e resulted in a dose-dependent prevention of neuronal cell death elicited by excitotoxicity, voltage-gated channel opening, and inhibition of endoplasmic reticulum Ca$^{2+}$-ATPase. These results indicate that DY-9760e can rescue neurons from various types of cell-toxic stimuli, which may contribute to attenuation of brain injury after cerebral ischemia.

Key words DY-9760e; calmodulin antagonist; neuronal cell death; intracellular Ca$^{2+}$; cerebral ischemia

Although the precise cellular mechanisms underlying ischemic damage remain to be clarified, the excessive release of glutamate and subsequent elevation of intracellular Ca$^{2+}$ levels are considered to be implicated in the pathogenesis of ischemic brain injury.1–9 It is proposed that the elevation of intracellular Ca$^{2+}$ levels can be brought about by a massive influx of extracellular Ca$^{2+}$ through voltage-gated1,5) or agonist-activated channels,6,7) and a release of Ca$^{2+}$ from intracellular store sites such as endoplasmic reticulum (ER) and mitochondria.8–10) Excessive elevation of Ca$^{2+}$ in neurons disrupts the ionic balance and activates various Ca$^{2+}$-dependent enzymes.

Calmodulin is a major Ca$^{2+}$-binding protein found mainly in the central nervous system, and it is implicated in a variety of physiological cell functions through the activation of calmodulin-dependent enzymes, such as phosphodiesterase, protein kinases, phosphatase, and nitric oxide synthase (NOS).11,12) Ca$^{2+}$ overload into neurons induced by ischemic insult may therefore aberrantly activate Ca$^{2+}$/calmodulin-dependent pathways and lead to irreversible cell damage. Indeed, it has been previously reported that a persistent increase in the concentration of Ca$^{2+}$-bound calmodulin, which is an active form of calmodulin, is associated with ischemic neuronal damage.13) Furthermore, calmodulin antagonists have been shown to protect against hypoxia/hypoglycemia in organotypic hippocampal cultures.14)

We have developed a competitive calmodulin antagonist, 3-[[4-((3-chloro-2-methylphenylmethyl)-1-piperazinyl)ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-indazole dihydrochloride 3.5 hydrate (DY-9760e), which exerts a cytoprotective action against the cell death induced by a Ca$^{2+}$ ionophore8) and ameliorates brain injury that occurs after transient and permanent focal ischemia in rats.16–19) Since DY-9760e does not alter cerebral blood flow (CBF) following focal ischemia,17) it may produce the neuroprotection against cerebral ischemia via mechanisms other than the improvement of CBF. To further clarify the neuroprotective properties of DY-9760e, using rat primary cultured neurons, we evaluated the effect of the drug on the neuronal cell death elicited by cell-toxic stimuli that mimic cellular events observed in cerebral ischemia. We show here that DY-9760e rescues neurons from cell death resulting from excitotoxicity, membrane-depolarization, and inhibition of ER Ca$^{2+}$-ATPase.

MATERIALS AND METHODS

Materials Veratridine was purchased from Biomol Research Labs (Plymouth Meeting, PA, U.S.A.). KCl was obtained from Nacalai Tesque (Kyoto, Japan); L-glutamate from Tokyo Kasei Kogyo (Tokyo, Japan); thapsigargin from SIGMA (St. Louis, MO, U.S.A.); N-methyl-D-aspartate (NMDA) from Research Biochemical Int. (Natick, MA, U.S.A.). DY-9760e was synthesized at Daiichi Pharmaceutical Co. (Tokyo, Japan).

Neuronal Cultures Primary cortical neurons were cultured from embryonic day 17–18 rat fetuses as described previously.20) Neurons were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with B27 serum replacement (Invitrogen, Carlsbad, CA, U.S.A.). The neurons were plated onto 96-well polyethyleneimine-coated culture plates at a density of 30000 cells/well. Experiments were performed after the neurons were cultured for 10 d.

Cell Death Assay The experiments were carried out in DMEM containing B27 supplement. DY-9760e was dissolved in 50 mM Sörensen buffer (pH 4.5), which was prepared from 50 mM glycine and 50 mM sodium chloride adjusted for pH with 1 N HCl, and applied to the cells 1 h prior to the treatment with various cell-death inducers. The neuronal cultures were subsequently exposed to toxic agents under the following conditions: 50 μM glutamate for 10 min; 100 μM NMDA for 15 min; 10 μM veratridine for 24 h; 90 mM KCl for 30 min; or 100 μM thapsigargin for 24 h. Cell death was assessed by measuring the amount of lactate dehydrogenase...
DY-9760e Protects Neurons from Excitotoxicity Following cerebral ischemia, the release of excess glutamate into the extracellular space triggers the opening of cation-permeable channels at NMDA receptor followed by Ca\(^{2+}\) influx into neurons.\(^1\text{-}^4\) We first investigated whether DY-9760e protects against neuronal cell death induced by glutamate and NMDA. The incubation of cortical neurons with 50 \(\mu M\) glutamate for 10 min caused significant neuronal cell death, as determined by LDH leakage from the neurons 24 h after the application (Fig. 1A). DY-9760e reduced the LDH leakage by 30, 50, 60 and 85\% at the concentrations of 0.01, 0.1, 1 and 10 \(\mu M\), respectively (Fig. 1A). In addition, DY-9760e dose‐dependently prevented the LDH leakage from cells 24 h after exposure to 100 \(\mu M\) NMDA for 15 min (Fig. 1B). These results indicate that DY-9760e protects against excitotoxic neuronal cell death.

DY-9760e Protects against Neuronal Cell Death Induced by Voltage-Gated Channel Opening Neuronal cell damage is initiated by not only the entry of Ca\(^{2+}\) through glutamate receptors, as described above, but also Ca\(^{2+}\) influx through voltage-sensitive channels upon membrane depolarization.\(^5\text{-}^7\) To determine the effect of DY-9760e on cell death caused by the activation of voltage-sensitive channels, we used two different membrane-depolarizing agents, veratridine and high KCl, that allow not only Na\(^{+}\), but also Ca\(^{2+}\) to enter into the cytoplasm through these channels.\(^21\text{-}^23\) Exposure of neurons to 10 \(\mu M\) veratridine for 24 h increased LDH leakage up to approximately 4.1-fold above that in the control; this leakage was significantly inhibited by treatment with DY-9760e at concentrations of 0.01—10 \(\mu M\) (Fig. 2A). Similarly, treatment with DY-9760e resulted in a dose-dependent (1—10 \(\mu M\)) reduction in LDH leakage from cells exposed to 90 \(mM\) KCl for 30 min (Fig. 2B). Thus, these data suggest that DY-9760e can protect neurons from cell death mediated by the activation of voltage-gated channels in response to membrane depolarization.

DY-9760e Inhibits Neuronal Cell Death Induced by Release of Sequestered Intracellular Ca\(^{2+}\) To explore the possibility that DY-9760e provides neuroprotection through intracellular sites of action, we treated neurons with thapsigargin, a compound that is known to cause an increase in cytosolic Ca\(^{2+}\) by blocking the ER calcium pump, Ca\(^{2+}\)-ATPase.\(^24\) As shown in Fig. 3, the incubation with 100 \(nM\) thapsigargin for 24 h led to neuronal cell death, and DY-9760e (0.1—10 \(\mu M\)) significantly blocked thapsigargin-induced cell death in a dose dependent manner.

DISCUSSION

We have previously reported that DY-9760e, a potent calmodulin antagonist, reduces the cerebral infarct volume and brain edema after transient and permanent focal ischemia in rats.\(^16\text{-}^19\) In this report, we provide evidence that DY-9760e can rescue neurons against cell death resulting from excitotoxicity, membrane-depolarization, and inhibition of ER Ca\(^{2+}\)-ATPase, all of which are known to increase intracellular Ca\(^{2+}\).

It is widely accepted that an excessive elevation of intracellular Ca\(^{2+}\) concentration plays a major role in ischemic...
cell injury in neurons. This increase is mainly caused by the influx of extracellular Ca\(^{2+}\) through receptor-operated or voltage-gated channels, and the release of sequestered Ca\(^{2+}\) from ER and mitochondria. Glutamate, a major excitatory neurotransmitter in the central nervous system, is a potential toxin leading to excitotoxicity when it is over-accumulated in the extracellular space. Glutamate-induced excitotoxicity is associated with an excessive influx of Ca\(^{2+}\) mainly via the NMDA receptor of glutamate. Some Ca\(^{2+}\) has been shown to enter through \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptor-gated channels in cultured neurons.

In addition to excitotoxicity, voltage-gated channel opening induced by veratridine, the voltage-sensitive Na\(^+\) channel activator, or high KCl, also causes neuronal cell death, which is brought about by massive Ca\(^{2+}\) influx. In this study, we found that DY-9760e protects neurons from cell death induced both by excitotoxicity with glutamate or NMDA and by activation of voltage-gated channels with veratridine or high KCl. Furthermore, the drug prevented a reduction in cell viability after exposure to thapsigargin, a specific inhibitor of ER Ca\(^{2+}\)-ATPase that mobilizes Ca\(^{2+}\) from intracellular stores. Thus, these results indicate that DY-9760e is protective against neurotoxicity elicited not only by influx of extracellular Ca\(^{2+}\) through voltage-gated channels or glutamate receptors but also by release of sequestered intracellular Ca\(^{2+}\) and suggest the possibility of an intracellular site of action.

There is evidence that calmodulin plays a critical role in ischemic brain injury as follows: 1) a persistent increase of Ca\(^{2+}\)-bound calmodulin, which is an active form, after ischemic insults; 2) significant up-regulation of calmodulin gene expression in the CA1 pyramidal cell layer after cerebral ischemia; 3) protection by calmodulin antagonists against hypoxic-hypoglycemia in organotypic hippocampal cultures; and 4) reduction in transient focal ischemia-induced brain injury by treatment with trifluoperazine, a calmodulin antagonist that is structurally different from DY-9760e. DY-9760e has been shown to prevent Ca\(^{2+}\)-ionophore-induced cell death in neuroblastoma cells with a more potent protective effect than that of W-7, a well-known calmodulin antagonist. Given the protection shown by DY-9760e against a variety of cell-death inducers, it is plausible that the amelioration of ischemic brain injury with this drug is mediated by calmodulin antagonistic action. Furthermore, with respect to calmodulin-mediated cell death, there are several intriguing reports: 1) PEP-19, a brain-specific protein and negative regulator of calmodulin, can inhibit apoptotic processes in PC12 cells following UV irradiation or staurosporine treatment; and 2) immunoreactivity for PEP-19 is dramatically reduced in the brains from patients with Alzheimer’s and Huntington’s disease. These data indicate that calmodulin may also contribute to neuronal loss in neurodegenerative diseases.

Calmodulin is implicated in a variety of cell functions by its interactions with various calmodulin-binding proteins. Among them, fodrin, a cytoskeletal protein lying under the plasma membrane in neurons, is degraded by calpain, a Ca\(^{2+}\)-dependent protease, after cerebral ischemia, thereby leading to membrane damage and finally cell death. Interestingly, it is reported that calmodulin facilitates the proteolytic degradation of fodrin by calpain, suggesting a role for calmodulin in regulating proteolysis by calpain. DY-9760e, which exerts no direct action on calpain, can indeed inhibit the degradation of fodrin after transient focal ischemia. Furthermore, intracellular Ca\(^{2+}\) overload after ischemic insult may induce the sustained activation of Ca\(^{2+}\)/calmodulin-dependent enzymes, such as NOS, calcineurin, and calmodulin kinase II (CaMKII), leading to irreversible cell damage. We have also demonstrated that DY-9760e inhibits not only purified neuronal NOS activity most potently but also NO production after exposure of neuroblastoma cells to Ca\(^{2+}\)-ionophore and cerebral ischemia/reperfusion in gerbils. The neuroprotection of DY-9760e may, therefore, be mediated at least in part by inhibition of NO production and fodrin degradation. Based on previous observations, together with present findings, we propose a scheme for the mechanism by which DY-9760e may exert neuroprotection by blocking Ca\(^{2+}\)/calmodulin-dependent pathways (Fig. 4).

In conclusion, DY-9760e can rescue neurons from various types of cell-toxic stimuli that increase intracellular Ca\(^{2+}\). Although further investigations are required to address which Ca\(^{2+}\)/calmodulin-dependent pathways play critical roles in inducing neuronal cell death, the protection of DY-9760e is likely to be mediated by blocking several Ca\(^{2+}\)/calmodulin-dependent pathways. Thus, drugs that inhibit aberrant activation of calmodulin-dependent pathways may have a therapeutic effect in the acute phase of cerebral ischemic damage.

REFERENCES
