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学位の種類	博　士（薬　学）
学位記番号	薬　第　４７０　号
学位授与年月日	平成16年1月21日
学位授与の要件	学位規則第4条第2項該当

学位論文題目

Inhibition of calmodulin as a new therapeutic strategy for cerebral ischemia: DY-9760e, a novel calmodulin antagonist, has a protective action against cerebral ischemic insults
(脳虚血障害治療の新戦略としてのカルモデュリン阻害：新規カルモデュリン拮抗薬DY-9760eの脳虚血障害に対する保護作用)

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論 文 内 容 要 旨

An excessive elevation of intracellular Ca^{2+} concentration plays an important role in pathological events after cerebral ischemia. Calmodulin is a major Ca^{2+} binding protein in the central nervous system. It has been shown that persistent increase of Ca^{2+}-bound calmodulin, which is an active form of calmodulin, is associated with neuronal damage after ischemia. Thus, Ca^{2+} overload in neurons may induce aberrant activation of Ca^{2+}/calmodulin signaling systems, resulting in neuronal damage. DY-9760e (Fig. 1), an indazole derivative, is a newly synthesized specific calmodulin antagonist that is more potent than W-7, a well-known calmodulin antagonist.

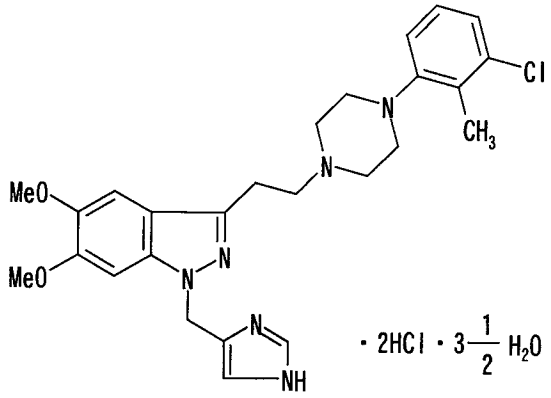


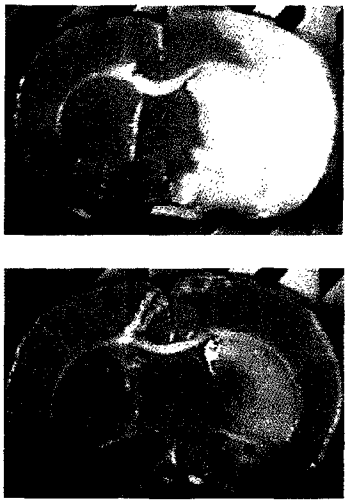
Fig. 1. Chemical structure of DY-9760e.
3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-indazole dihydrochloride 3 1/2 hydrate.

Furthermore, DY-9760e can protect cells from cytotoxicity induced by the Ca^{2+}-ionophore that causes an increase in intracellular Ca^{2+}, and its protective effect is more potent than that of W-7. These findings suggested that inhibition of calmodulin is useful for neuroprotection. Therefore, the author evaluated effects of DY-9760e in cerebral ischemia models and analyzed mechanism to provide its neuroprotective action.

First, effect of DY-9760e was evaluated in rats with permanent focal ischemia. Intravenous administration of DY-9760e (0.5 mg/kg/h for 6 h) significantly reduced infarct volume caused by occlusion of the middle cerebral artery (MCA). The neuroprotective effect was still observed even when the administration was started at 3 h after MCA occlusion. Furthermore, application of a loading dose of 0.3 mg/kg/h for 2 h followed by infusion at 0.1 mg/kg/h for 22 h allowed DY-9760e to be effective, in contrast to its failure to be protective at 0.1 mg/kg/h for 24 h without the loading dose. Thus, prolongation of continuous infusion preceded by a loading dose is an efficacious dosing regimen for this compound, especially with the use of a low infusion rate. It is known that cerebral ischemia produces more severe brain injury in spontaneously hypertensive rats (SHRs) than in any normotensive strain. DY-9760e showed a protective effect against permanent focal ischemia in SHRs.

Next, the author investigated whether DY-9760e provides protection in a model with ischemia/reperfusion, which leads to development of severe brain edema. DY-9760e attenuated the cerebral infarction (Fig. 2) and brain edema

formation after a transient MCA occlusion, and its neuroprotective effect was also confirmed by magnetic resonance imaging. Thus, DY-9760e was protective against not only permanent focal ischemia but also transient focal ischemia.



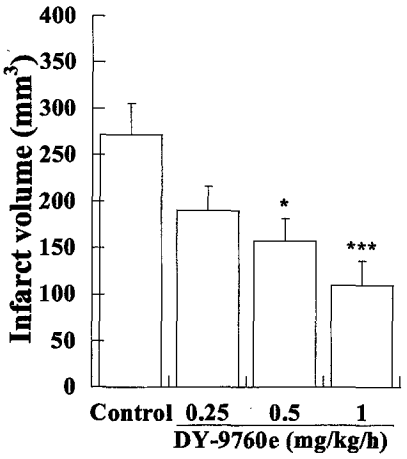


Fig. 2. Effects of DY-9760e on cerebral infarct volume in transiently MCA-occluded rats. Images are representative 2,3,5-tripheyltetrazolium chloride-stained coronal brain sections: above, vehicle; below, DY-9760e (1 mg/kg/h).

To investigate whether calmodulin is involved in the process of brain damage, effect of trifluoperazine, a calmodulin antagonist structurally unrelated to DY-9760e, on brain edema caused by transient MCA occlusion was examined. Trifluoperazine, as well as DY-9760e, attenuated development of brain edema following transient focal ischemia, suggesting involvement of calmodulin in the brain edema following cerebral ischemia. Fodrin, a cytoskeletal calmodulin-binding protein, is degraded by calpain, a Ca^{2+}-dependent protease, after cerebral ischemia. Calmodulin has been shown to stimulate its degradation by calpain. DY-9760e reduced fodrin breakdown after transient focal ischemia in a dose-dependent manner. Because DY-9760e did not directly affect calpain activity *in vitro*, the inhibition of fodrin breakdown is due to its calmodulin-antagonistic effect for fodrin. In addition to the effect of DY-9760e on fodrin degradation, the author and his colleagues have demonstrated that DY-9760e inhibits nitric oxide production in cells exposed to Ca^{2+}-ionophore and animals subjected to cerebral ischemia, and release of arachidonic acid from C6 glioma cells elicited by activation of cytosolic phospholipase A_2. DY-9760e had no effect on cerebral blood flow in the cortex. The neuroprotective action of DY-9760e is likely to be mediated by blocking several Ca^{2+}/calmodulin-dependent pathways in the process of brain damage.

Since DY-9760e and trifluoperazine attenuated development of brain edema following transient focal ischemia, the calmodulin antagonists may affect a change in the blood-brain barrier (BBB) integrity after transient focal ischemia. In fact, DY-9760e ameliorated the BBB disruption induced by cerebral ischemia/reperfusion. Furthermore, DY-9760e and trifluoperazine reduced TNF- α -induced hyperpermeability of inulin through a cultured brain microvascular endothelial cell monolayer *in vitro*. These results suggest that the protective effect of calmodulin

antagonists, including DY-9760e and trifluoperazine, is mediated in part by inhibition of enhanced BBB permeability after ischemic insults.

In the present study, the author demonstrated that DY-9760e is a neuroprotective compound against cerebral ischemia. As summarized in Fig. 3, the calmodulin antagonist inhibits calmodulin-dependent pathways, which account for neuronal cell death. The author also demonstrated that DY-9760e rescued neurons from brain damage including brain edema and BBB disruption. However, further studies are required to define mode of action of DY-9760e on the inhibition of brain edema and BBB disruption.

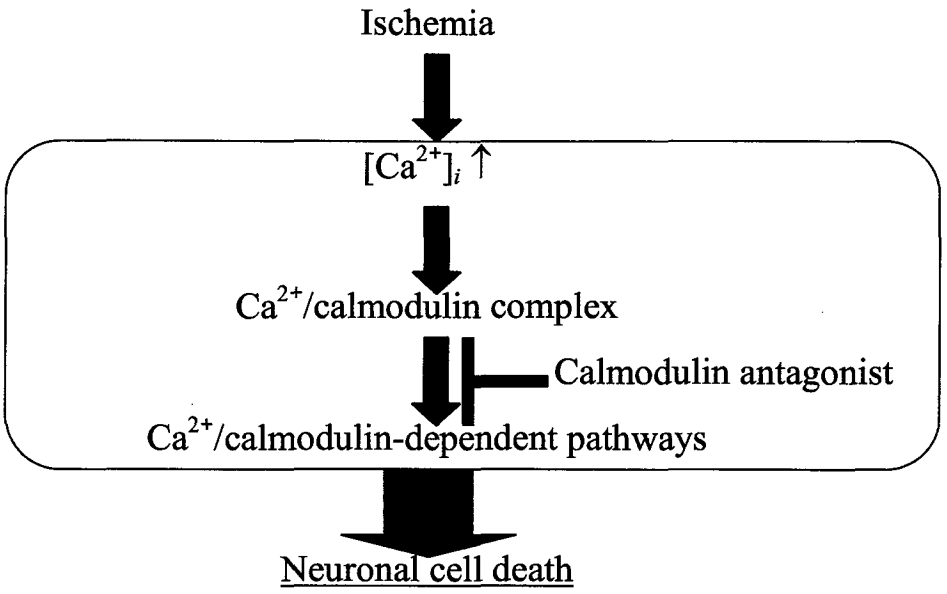


Fig. 3. Ca^{2+}/calmodulin hypothesis for induction of neuronal cell death.

In this context, DY-9760e is not only a potential, therapeutic drug for treatment of acute stroke patients but also prototype drug to define functional relevance of calmodulin in the mechanisms of neuronal death following brain ischemia.

審査結果の要旨

現在の科学レベルでは，脳虚血によって障害された中枢神経細胞を再生することは難しい。そのため，脳虚血発症後の損傷組織の拡大を阻む脳保護薬の出現が待望されている。しかし，虚血による神経細胞死の要因としては受容体・酵素を含め様々なものが報告されており，脳保護薬開発上，標的を絞り込むことを困難にしている。佐藤俊之氏は神経細胞死の引き金となるCa^{2+}流入と，細胞死に直接関わる様々な酵素との間に位置するCa^{2+}結合蛋白のcalmodulinに着目し，その活性化を抑制することで虚血性神経細胞死を阻止できるとのこれまでに無い仮説を立て，その実証のために末梢投与可能な新規calmodulin拮抗薬DY-9760eを創製した。同氏はDY-9760eがラット脳虚血モデルで脳保護作用を示すこと，その脳保護作用がcalmodulin拮抗に基づくことを明らかにし，脳虚血障害克服の標的としてcalmodulinが妥当であることを本研究で示した。

本論文では初めに，DY-9760eを持続静注することによりラット脳虚血モデルで生じる脳梗塞の容積が減少することを示した。また脳浮腫の軽減作用を認めることも明らかにし，さらにDY-9760eと化学構造の異なるcalmodulin拮抗薬のtrifluoperazineにも同様の効果が認められることを示した。次に，DY-9760eの脳保護作用は脳血流増加に因るものではなく，calmodulin阻害による神経細胞に対する直接的な保護作用に因ることを神経細胞障害マーカーのfodrin分解産物の減少より明らかにした。また，DY-9760eが虚血による血液脳関門の機能破綻を抑止することを脳虚血モデルで明らかにして，その作用が血管内皮細胞のバリア機能保全によることを示した。以上の結果からcalmodulin拮抗薬は，直接的な神経細胞保護作用と血液脳関門機能保全による虚血性２次障害の防止作用を併せ持つことが示された。

本研究の知見は，calmodulin拮抗薬が脳保護薬として有用である可能性を示唆するという重要かつ新規性に富む研究であると言える。

よって，本論文は博士(薬学)の学位論文として合格と認める。