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Review

Ca^{2+} channel antagonists and neuroprotection from cerebral ischemia

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Abstract

Stroke is the third leading cause of death and the main disabling neurologic disease. The finding in experimental studies that neuronal death does not occur immediately after ischemic injury has encouraged the development of neuroprotective agents. Various Ca^{2+} channel antagonists, that is, L-type-selective or non-selective derivatives from classical Ca^{2+} channel antagonists, have been examined for their ability of neuroprotection through improvement of cerebral blood circulation or inhibition of Ca^{2+} overload induced by excessive glutamate release. Although some of the antagonists showed efficient neuroprotection in animal models, systemic hypotension limited the utility of these drugs, and none of the compounds showed beneficial effects in treatments for acute ischemic stroke in clinical trials. Drugs other than Ca^{2+} channel antagonists developed on the basis of the glutamate–Ca^{2+} overload hypothesis were shown also to lack clinical benefit. Recently, some mechanisms have been proposed to interpret neuronal death in relation to hyperexcitability or apoptosis after ischemic insult. In these hypotheses, activation of the Ca^{2+} channel types selectively expressed in neuronal tissues is proposed as a critical step of the pathways toward neurodegeneration. Thus, it is increasingly recognized that developing highly selective compounds for neuronal Ca^{2+} channels is not only important for treatment of stroke but also for elucidation of mechanisms that underlie neurodegeneration.

Keywords: Ca^{2+} channel antagonist; Neuroprotection; Ischemia; Voltage-dependent Ca^{2+} channel

1. Introduction

In late 1960s, nifedipine, verapamil and diltiazem, emerged as a novel group of drugs 'Ca^{2+} channel antagonist', because of their selectivity in blocking voltage-dependent Ca^{2+} channels (VDCC) (Fleckenstein, 1983). Ca^{2+} channel antagonists were developed originally for the treatment of angina pectoris by dilation of coronary arteries. However, they proved beneficial also for hypertension, ischemic heart disease, and cardiac arrhythmia (Triggle, 1991).

Stroke, comprised of embolisms, thromboses, or hemorrhages in the brain, interrupts vital blood supply and thereby induces localized ischemic damage in brain tissues. Since stroke is a dysfunction of cerebral blood circulation, the remarkable utility of the Ca^{2+} channel antagonists in cardiovascular diseases had encouraged the examination of therapeutic potentials of these drugs in cerebrovascular dysfunctions. Although many of these compounds, synthesized to dilate cerebral artery, blocked Ca^{2+} channels in vascular smooth muscle cells, their clinical effects were disappointing.

From the view point of the glutamate–Ca^{2+} overload neurotoxicity hypothesis (Choi, 1988; Siesjo and Bengtsson, 1989), inhibition of excessive Ca^{2+} influx into neurons was considered to be important for neuroprotection. It is natural to apply Ca^{2+} channel antagonists directly to neuronal tissue, considering the prominent distribution of VDCC in the brain and the physiological importance of Ca^{2+} in neurons. However, no compounds were proven to be effective in clinical trials of more than 10 years. Therefore, not so many Ca^{2+} channel antagonists were newly synthesized as neuroprotective drugs, and researchers turned their attention to new candidates based upon glutamate–Ca^{2+} overload hypothesis such as glutamate receptor (NMDA, AMPA) antagonists, radical scavengers, Na^{+} overload inhibitors, calpain inhibitors, nitric oxide synthetase inhibitors. Although they showed promising results in animal studies, many compounds developed from the cascade of glutamate–Ca^{2+} overload hypothesis

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also proved to be ineffective or caused side effects in clinical trials.

Recently, hyperexcitability and apoptosis have been proposed as novel mechanisms that lead to delayed neuronal death, which has encouraged revision of glutamate–Ca^{2+} overload hypothesis. In this article, we describe possible relationships between such mechanisms and neuronal VDCCs, and discuss the potential of Ca^{2+} channel antagonists becoming neuroprotective agents. We also review Ca^{2+} channel antagonists developed so far and their problems as neuroprotective agents in the clinic.

2. Neuronal damage by cerebral ischemia

2.1. Types of ischemic neuronal death

2.1.1. Blood flow level and types of neuronal death

Eighty percent of strokes are due to blood clots. After onset of stroke, blood clots form focal ischemia that evoke neuronal damage in restricted areas of brain. Three types of neuronal death have been distinguished based on the level of residual blood flow in the ischemic area (Fig. 1). (1) At the center of ischemic region where the cerebral circulation is completely arrested, irreversible cell damage occurs in several minutes. In the core area, neurons cannot be saved by medical treatments after the onset of stroke. (2) In the area surrounding the center, the ischemia is incomplete because of the presence of perfusion from collateral vessels. This region is called 'penumbra' (Astrup et al., 1981), where reduced blood flow falls to the level below the threshold for electrical failure and above the threshold for energy failure. In penumbral areas, the state of neurons are described by electric silence with normal or only slightly elevated K^+ concentration in extracellular space. Experimental results suggest that such moderate ischemia does not lead to immediate neuronal damage. Neurons under these conditions are able to survive for several hours (Hossmann, 1994) so that appropriate medical/pharmacological treatments can reverse neuronal death. (3) Another type of neuronal death occurs in a region apart from the ischemic area. It has been reported that ischemic neuronal death in the striatum causes neuronal degeneration in the ipsilateral substantia nigra (Nagasawa and Kogure, 1990; Tamura et al., 1990). Retrograde neuronal degeneration in the thalamus after cortical infarcts produced by ischemia has been also reported (Iizuka et al., 1990). The neuronal deaths in non-ischemic area occurs 1–2 weeks after the onset of ischemia. Activity of neuronal networks may play a vital role in this type of neuronal death that is also an important target of drug therapy.

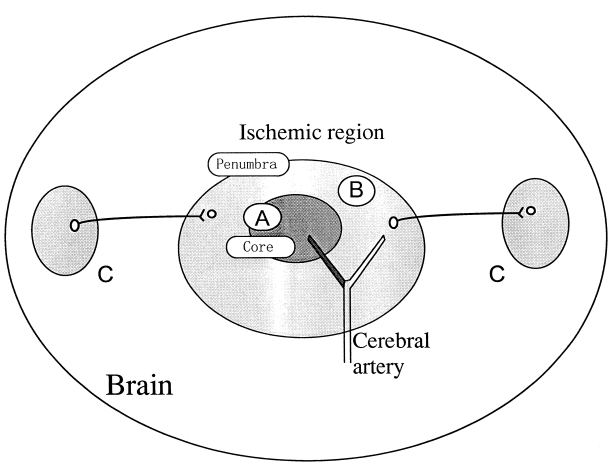

Fig. 1. A Diagram showing three types of neuronal death after focal ischemia. Neuronal death in ischemic core region (A) where blood flow is completely stopped; in penumbra area surrounding ischemic core (B) where collateral circulation supplies vital flow to neurons for several hours but certain triggers may initiate cascades of neurodegeneration; and in non-ischemic areas (C) where neurons are connected to those in the ischemic area with synapse(s).

2.1.2. Delayed neuronal death

If ischemia is severe and persists for a long time, neurons become necrotic and die rapidly. On the other hand, if the blood flow is recovered soon after global ischemia, nothing immediate happens to brain neurons; whose appearance, activity and energy status remain normal. However, delayed neuronal death is observed selectively in vulnerable areas such as hippocampus several days later (Kirino, 1982; Pulsinelli et al., 1982). Delayed death is less extensive in other regions such as cortical area (Pulsinelli et al., 1982) than in hippocampus, because of the scattered distribution of vulnerable neurons. Since neurons in the penumbra of focal ischemia or in certain non-ischemic areas survive for more than several hours, it is generally accepted that the same mechanism of neuronal death is involved in these areas. The slow development of delayed neuronal death enables appropriate treatments including drug therapies to rescue these neurons in ischemia.

2.2. Mechanisms of neuronal death

As mentioned above in Section 2.1.1, multiple types of neuronal death triggered by dysfunction of cerebral circulation take place in focal ischemia. Subsequent to the primary trigger at the onset of stroke, deterioration of microcirculation still continues to threaten the survival of neurons for several hours or days. In parallel, intrinsic mechanisms proceed in neurons to induce neuronal death. We separately discuss the two delayed mechanisms in neuronal death.

2.2.1. Deterioration of microcirculation

After onset of focal ischemia, the blood flow level decrease in the microvessels of the penumbra, which can facilitate secondary clot formation. Although neurons still survive in the penumbral area, there remains a threat of secondary ischemia which damages the neurons.

Even if restoration of blood flow is achieved through treatment by tissue plasminogen activator or the spontaneous clearance of an obstructing clot, penumbral tissues suffer from 'delayed postischemic hypoperfusion', in which the blood flow reaches minimum after 1–4 h of reperfusion and persists for 24 h (Hossmann and Kleihues, 1973). It has been proposed that the activation of blood cells (platelets and leukocytes) and their interaction with endothelial cells contribute to the impairment of microcirculation and other reperfusion injuries.

In the case of hemorrhagic stroke, patients are threatened by subarachnoid or, sometimes, intracerebral hemorrhage during the acute phase, and by delayed cerebral ischemia due to cerebral vasospasm, which generally occurs 1 or 2 weeks after hemorrhage (Saito et al., 1977).

2.2.2. Damage intrinsic to neurons

2.2.2.1. Glutamate and Ca^{2+}. The glutamate–Ca^{2+} neurotoxicity hypothesis (Choi, 1988; Siesjo and Bengtsson, 1989) has been accepted for more than 10 years. In the hypothesis, it is proposed that excessive amount of excitatory neurotransmitters, particularly glutamate, released in synaptic clefts is neurotoxic. Glutamate causes pathologic elevation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) that activates Ca^{2+}-dependent enzymes and generates toxic levels of nitric oxide and free radicals to damage macromolecules and cell membranes. Furthermore, accumulation of free Ca^{2+} in cells causes mitochondrial calcium overload and cessation of ATP production, which also leads to neuronal death. Key roles of the excessive glutamate release and the following Ca^{2+} influx during ischemic neuronal death have been experimentally confirmed by many investigators (reviewed by Rothman and Olney, 1995). However, it is not established yet exactly what intracellular cascades proceed during neuronal death. In fact, experimental observations suggest that the delayed neuronal death cannot be explained only by the original hypothesis. After restoration of blood flow, elevated $[Ca^{2+}]_i$ returns to normal levels (Silver and Erecinska, 1992). Glutamate accumulation in the neuronal microenvironment is also cleared within minutes during reperfusion by glutamate transporters (Obrenovitch and Richards, 1995). Furthermore, although the penumbra is the most receptive to cerebroprotection with glutamate receptor antagonists, extracellular glutamate levels may not reach critical levels in the region (Obrenovitch and Richards, 1995). Thus, several lines of evidence suggest that excessive glutamate release and $[Ca^{2+}]_i$ elevation is only transient during ischemia, and act as a trigger for delayed death.

In this context, Szatkowski and Attwell (1994) proposed an interesting hypothesis about a dual role of glutamate: triggering and execution of neuronal death. Excessive glutamate release during ischemia activates NMDA receptors to trigger long term potentiation of synaptic transmissions. As a consequence, even normal levels of glutamate release may activate neural networks with a higher efficiency after ischemia. This may further cause abnormal excitation (Kawai et al., 1995), which leads to the greatly enhanced energy demands and intracellular Ca^{2+} accumulation, and eventually to neuronal death. If this is the case, the toxicity of glutamate and Ca^{2+} is not stemmed from Ca^{2+} overload during ischemia, but from the widespread and persistent strengthening of neural transmission after ischemia. The hypothesis is supported by the observations that surgical removal or colchicine destruction of excitatory inputs protects hippocampal neurons from ischemia (Wieloch et al., 1985; Johansen et al., 1986). The mechanism of neuronal loss in non-ischemic areas remote from ischemic core may be explained by this hypothesis. It cannot be denied that neuroprotection by glutamate antagonists is due to their anticonvulsive effects (Dingledine et al., 1990), which suppress hyperexcitability after ischemia.

2.2.2.2. Protein synthesis and apoptosis. According to Hossmann (1994), selective neuronal loss occurs even at flow values below 0.80 ml g^{-1} min^{-1} when ischemia lasts a few hours. The value is far above the threshold of pannecrosis (0.12 ml g^{-1} min^{-1}) and that of glutamate release (0.20 ml g^{-1} min^{-1}). Interestingly, a threshold for inhibition of protein synthesis is about 0.55 ml g^{-1} min^{-1}. It is possible that the disturbance of protein synthesis, such as shortage of neurotrophins, is involved in the delayed neuronal death.

By contrast, protein synthesis and gene expression specific for neuronal death have been proposed. Inhibitors of protein synthesis, cycloheximide (Goto et al., 1990) and anisomycin (Shigeno et al., 1990) protected neurons from ischemia, suggesting the involvement of 'killer proteins'.

Accumulating evidence supports the involvement of apoptosis in delayed neuronal death. DNA fragmentation was observed in gerbil hippocampus after transient ischemia (Okamoto et al., 1993). Increased expression of p53, a protein responsible for induction of apoptosis, in ischemic brain tissues has been reported (Chopp et al., 1992). Furthermore, p53 knock-out mice exhibited ischemic damage significantly less than normal mice following middle cerebral artery occlusion (Crumrine et al., 1994). It has also been reported that overexpression of an inhibitory factor of apoptosis, Bcl-2, protected neurons from ischemic lesions (Martinou et al., 1994; Linnik et al., 1995), and that non-vulnerable neurons in the ischemic brain showed increased expression of Bcl-2 (Chen et al., 1995).

3. Functional aspects of neuronal Ca^{2+} channels

3.1. Functional and molecular diversity of Ca^{2+} channels

VDCCs serve as the only link to transduce depolarization into activities controlled by excitation in various

cellular processes including neurosecretion. The basis of this pivotal role of Ca^{2+} channels is the transient increase of $[Ca^{2+}]_i$. Ca^{2+} channels, together with Na^+ and K^+ channels, are also electrogenic. VDCCs are diverse in both biophysical and pharmacological properties. It has been recognized that there exist multiple VDCC types (L-, N-, P-, Q-, R- and T-type) that can be distinguished by their functional properties such as time- and voltage-dependent kinetics, single-channel conductance and sensitivity to different pharmacological agents (see review by Mori et al., 1996) (Table 1). L-type VDCCs are high voltage-activated, sensitive to dihydropyridines, and are found in virtually all excitable tissues and in many non-excitable cells. They trigger excitation–contraction coupling in skeletal muscle, heart and smooth muscle and control hormone or transmitter release from endocrine cells and some neurons. The N-type and P-type channels are high-voltage activated Ca^{2+} channels largely restricted to neurons, and are selectively blocked by ω-conotoxin GVIA and by ω-agatoxin IVA, respectively. Influx of Ca^{2+} through N- and P-type channels controls neurotransmitter release. Additionally, P-type channels probably play an essential role in inducing long term depression in cerebellar Purkinje neurons. Neuronal high voltage-activated Ca^{2+} channels also include Q- and R-types. Q-type is different from P-type only in sensitivity to ω-agatoxin IVA, but R-type is distinguishable from other high voltage-activated channels in T-type-like rapid inactivation and susceptibility to blockade by low concentrations of Ni^{2+}. T-type Ca^{2+} channels are low voltage-activated Ca^{2+} channels that have been implicated in repetitive firing and pacemaker activity in heart and neurons.

Over the last decade, there have been rapid advances in biochemical and molecular biological characterization of VDCCs. VDCC is expressed as a complex of the $\alpha 1$ subunit, which is both the essential channel moiety, and the receptor for dihydropyridines and other Ca^{2+} channel antagonists, and the auxiliary subunits α_2/δ, β, and γ. Recent evidence suggests that $\alpha 1$ subunits are encoded by at least nine distinct genes: α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, α_{1F} (Bech-Hansen et al., 1998; Strom et al., 1998), α_{1G}, α_{1H}, and α_{1S}, seven of which are known to be expressed in neuronal tissues. To date, four distinct genes have been reported for β, and two for γ subunits. Different molecular combination of the main $\alpha 1$ subunit, that determines the type of VDCC, with different auxiliary subunits may give rise to functional variability of particular types of VDCC.

The L-type VDCC in vascular smooth muscle cells responsible for vasoconstriction are well characterized, and most of the Ca^{2+} channel antagonists available in pharmacological treatments are selective to the L-type. Since numerous extensive reviews have already been written on the L-type channel and the action of the classical Ca^{2+} channel antagonists including dihydropyridines, we focus on neuronal VDCCs and their blockers in this review.

3.2. Diverse Ca^{2+} entry patterns generated by neuronal VDCCs

Since VDCC-types have distinct ion permeation properties, voltage dependence and kinetics of activation and inactivation gating, it is likely that Ca^{2+} influx induced by the same strength and duration of synaptic stimulation is highly diverged among VDCC types in functional parameters such as amplitudes and time courses. Patterns of $[Ca^{2+}]_i$ transient and Ca^{2+}-dependent depolarizing potentials through specific VDCCs may also depend on the subcellular localization of the channels because VDCC subtypes are differentially localized in individual neurons. Subcellular distribution of the N-type is complementary to the distribution of the L-type in the central nervous system (Westenbroek et al., 1990, 1992). In hippocampal pyramidal neurons, the L-type VDCCs are observed in cell bodies and concentrated at the base of the major dendrites (Westenbroek et al., 1990), whereas the N-type are localized primarily in apical dendrites (Westenbroek et al., 1992; Mills et al., 1994). Differential distribution of VDCC types with distinct functional characteristics may give rise to variation in susceptibility of neurons to ischemic damages.

3.3. Neurotransmitter release

VDCCs distributed in nerve terminals trigger neurotransmitter release by exocytosis. Usage of pharmacologi-

Table 1
Molecular classification of Ca^{2+} channel α_1-subunits

Nomenclature	Tissue distribution	Functional type	Selective blockers
α_{1S}	skeletal muscle	L	dihydropyridine
α_{1C}	ubiquitous (heart, brain, aorta, lung, fibroblast)	L	dihydropyridine
α_{1D}	brain, endocrine tissues	L	dihydropyridine
α_{1F}	retina	L	dihydropyridine
α_{1A}	brain, neuro-muscular junction	Q (P?)	ω-agatoxin IVA
α_{1B}	brain, sympathetic nerve terminal	N	ω-conotoxin GVIA
α_{1E}	brain	R?	Ni^{2+} (< 100 μM)
α_{1G}	brain, heart	T	mibefradil?
α_{1H}	liver, kidney, heart, brain	T	mibefradil

cal tools (blocking agents) alone and in combination has served to dissect the contribution of each Ca^{2+} channel type to neurotransmitter release. Most interestingly, experiments have revealed the variable extent of contributions by different Ca^{2+} channels in triggering neurotransmission, depending on the types of neuronal cells and synapses formed by them. It is generally accepted through numerous investigations using dihydropyridines that L-type Ca^{2+} channels make only minor contribution to neurotransmitter release in the brain. In biochemical studies of synaptosomes loaded with radiolabeled transmitters, glutamate release is mediated by ω-agatoxin IVA sensitive P-type channels (Turner et al., 1992). In the study with hippocampal slices (Gaur et al., 1994), the releases of aspartate and GABA (γ-aminobutyric acid) are mediated partially by P-type or ω-conotoxin MVIIC-sensitive Q-type. ω-Conotoxin GVIA sensitive N-type does not participate in these neurotransmitter releases. On the other hand, noradrenaline release seems to be mediated by N-type as well as P/Q-type. In electrophysiological studies using the patch clamp method on slice preparations (Takahashi and Momiyama, 1993), the contribution of P- and N-type to glutamate-induced transmission in hippocampus is 46 and 12%, respectively, whereas the contributions of P- and N-type Ca^{2+} channels to GABA release are 73 and 21% in cerebellar synapses, and 55 and 20% to glycine release in spinal synapses, respectively. Thus, experimental observations indicate that significance of each VDCC type in controlling transmitter release differ among types of neurons and synapses.

3.4. Other Ca^{2+}-dependent intracellular responses

Synaptic transmission induces postsynaptic Ca^{2+} influx into neurons, where Ca^{2+} acts as a second messenger that mediates a wide range of cellular responses. As reviewed by Ghosh and Greenberg (1995), Ca^{2+} bound to calmodulin stimulates the activity of a variety of enzymes, including Ca^{2+}-calmodulin-dependent kinase and type I Ca^{2+} sensitive adenylate cyclase. $[Ca^{2+}]_i$ increase also activates protein kinase C and Ras signalling pathway (Fig. 2). The enzymes that transduce Ca^{2+} signals affect long-lasting neuronal responses that require changes in gene expression such as axonal growth, synaptic plasticity and cell survival. In translocation of calmodulin that activates the transcription factor CREB (cAMP response element binding protein) through phosphorylation of serine residue by Ca^{2+}-calmodulin-dependent kinase IV, Ca^{2+} entry systems such as L-type VDCC and NMDA receptors are able to cause mobilization of calmodulin, whereas N- and P/Q-types are not (Deisseroth et al., 1998).

Ca^{2+} entry pathways distributed in different subcellular localization may evoke local Ca^{2+} signalling specialized for particular cellular responses. In this context, it is

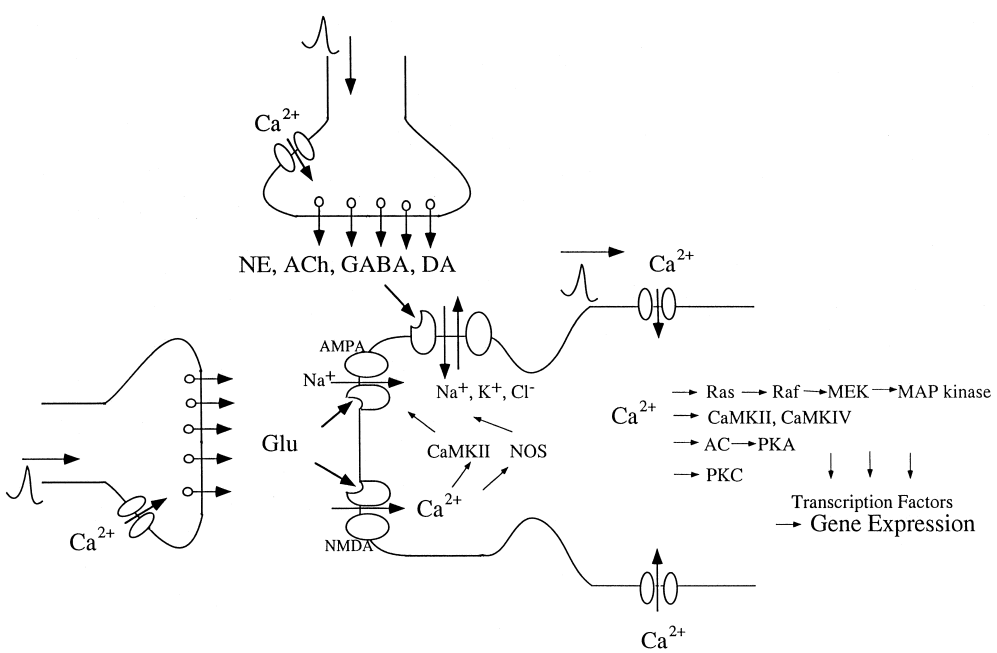

Fig. 2. Role of Ca^{2+} channels and Ca^{2+} in neuronal functions. Presynaptic VDCCs control the release of neuronal transmitters, not only glutamate but also other transmitters including acetylcholine (ACh), noradrenaline (NE), dopamine (DA), and GABA, which determine the excitability of the postsynaptic neuron. Influx of Ca^{2+} through NMDA receptor channels into postsynaptic neurons mediates neuroplasticity by the activation of calmodulin kinase II (CaMKII) and/or nitric oxide (NO) generation by calmodulin-dependent NO synthetase (NOS). In addition, Ca^{2+} binding to calmodulin stimulates a variety of enzymes, including the Ca^{2+}-calmodulin-dependent protein kinase (CaMK) II, IV, the type I Ca^{2+}-sensitive adenylate cyclase (AC-1). Increase in $[Ca]_i$ also activates protein kinase C (PKC) and Ras signaling pathways. These enzymes transduce the Ca^{2+} signal and cause long-lasting neuronal responses such as axonal growth, synaptic plasticity and cell survival that are mediated via changes in gene expression.

interesting to note that the L-type facilitation channel triggered much greater secretion for a given size of Ca^{2+} current than the N- and P-type channels in chromaffin cells (Artalejo et al., 1994). Highly localized, but small $[Ca^{2+}]_i$ transients via VDCCs in the brain neurons, that are almost undetectable using conventional electrophysiological methods, may be sufficient to elicit certain physiological functions.

4. Ca^{2+} channel blockade as a potential therapeutic strategy for stroke

Developments of neuronal death described in Section 2 involve processes that can be connected to Ca^{2+} influx through VDCCs. This suggests inhibition of VDCC as a target for therapy of stroke.

4.1. Improvement of cerebral circulation

In the penumbral area, even after reperfusion, decrease in cerebral circulation persists. Dilation of collateral blood vessels and enhancement of circulation aid in rescuing neurons from the damages. Blockade of VDCCs in smooth muscle cells of cerebral artery that causes vasodilation is easily achieved by Ca^{2+} channel antagonists. It has been reported that many Ca^{2+} channel antagonists preferentially antagonize the action of constrictors on the cerebral vascular smooth muscle, compared with non-cerebral vascular smooth muscle (Allen and Banghart, 1979; Shimizu et al., 1980). In addition, high affinity binding of Ca^{2+} channel antagonists (especially dihydropyridines) to the inactivated state of L-type VDCC (Bean, 1984) indicates that the drugs inhibit contraction of smooth muscle cells in a depolarizing environment more efficiently than contraction in a normal condition. This characteristic of the Ca^{2+} channel antagonists has the advantage of selective interaction of the drug with cerebral arteries in the ischemic penumbral area.

However, vasodilation does not necessarily lead to neuroprotection. Hypotension induced by Ca^{2+} channel antagonists even worsens the blood supply to the ischemic area. Furthermore, in the ischemic area where cerebral blood flow regulation is impaired, vasodilation is physiologically at its highest (Besson and Bogousslavsky, 1991). Inversely, in non-ischemic areas where autoregulation is preserved, decrease in blood pressure induces vasodilation to increase blood flow. Therefore, vasodilation therapy by Ca^{2+} channel antagonists may result in an intracerebral steal, which worsens cerebral damages.

Activation of platelets, granulocytes and other blood cells responsible for reperfusion injury, requires increase in their $[Ca^{2+}]_i$. However, there is little study on VDCCs of blood cells and endothelial cells, and on the effects of Ca^{2+} channel antagonists on these cells.

4.2. Blockade of neuronal Ca^{2+} channels

A number of peptide toxins show blockade selectivity to certain types of neuronal VDCCs. The benefit of direct block of neuronal VDCC for neuroprotection from ischemia has not been studied extensively so far. However, considering the physiological significance of VDCC, as discussed above, it is highly possible that activities of neuronal VDCCs contribute to development of delayed neuronal death after reperfusion. Recently, neuroprotective effects of an N-type selective blocker SNX-111 (ω-conotoxin MVIIA) (Valentino et al., 1993) has attracted much attention of researchers to encourage their studies on these peptide toxins.

4.2.1. Inhibition of neurotransmitter release

Major therapeutic strategies involve prevention or reduction of excessive release of neurotoxic glutamate from nerve terminals in ischemia by blockade of VDCCs. However, glutamate release during ischemia is due to reversed transport of glutamate by transporters from neurons and glial cells, and not due to Ca^{2+}-dependent exocytosis (Szatkowski et al., 1990). Therefore, neuronal VDCCs do not play a major role in this glutamate release, but in that after recovery from ischemia.

Since a dual role of glutamate functioning, as a trigger during ischemia and as an executor after ischemia in neuronal death, was proposed as described in Section 2, the control of hyperexcitability after recovery from ischemia has become a very important criteria in pharmacological treatments. It is possible that the neuroprotective effects of phenytoin (Cullen et al., 1979) or pentobarbital (Hallmayer et al., 1985) in cerebral ischemia are due to their ability to suppress hyperexcitability. Phenytoin has been reported to block low-threshold Ca^{2+} currents in neuroblastoma (Twombly et al., 1988) and to inhibit depolarization-induced Ca^{2+} influx into synaptosomes (Sohn and Ferrendelli, 1976), while pentobarbital has been known as a blocker of Ca^{2+} currents of dorsal root ganglion neurons (Gross and Macdonald, 1988) and Ca^{2+} influx into synaptosomes (Leslie et al., 1980). These compounds may suppress hyperexcitability through inhibition of neuronal VDCCs which reduces neurotransmitter release.

There are few reports about neuroprotection using compounds selective to VDCCs in nerve terminal. Furthermore, the type(s) of VDCC to be inhibited and the transmitters involved are unclear. At a concentration (50 nM) that significantly blocks N-type, a N-type-selective blocker ω-conotoxin MVIIA showed neuroprotection in hippocampal slices from anoxia (Pringle et al., 1996), strongly suggesting that neuronal death is mediated by N-type channels. Although it is highly possible that the protective effect was mediated by presynaptic modulation of transmitter release, other mechanisms involving postsynaptic N-type VDCCs is possible, as well. Interestingly, ω-con-

otoxin MVIIC, a potent inhibitor of glutamate release, did not show neuroprotection (Valentino et al., 1993) (see Section 5).

It is generally accepted that presynaptic VDCCs control the release of transmitters throughout the brain. The same type of VDCC mediate the release of not only excitatory transmitters but also inhibitory transmitters (see Section 3). Furthermore, transmitters inhibit neuronal VDCCs and suppress neurotransmitter release through the activation of G-proteins. It is therefore not conclusive that the block of VDCC leads to enhancement or suppression of neuronal excitation. However, experimentally, many compounds that show antagonistic action on VDCC seem to suppress hyperexcitability (Meyer, 1989), although this suppression can be mediated via their additional inhibitory effects on Na^+ channels and consequent blockade of axonal and dendritic conductance.

T-type VDCC may play an important role in repetitive firing of rhythmic neurons which generates propagating waves of excitation throughout the brain where synaptic transmission is enhanced after ischemia. Therefore, blockade of T-type VDCC is one of the interesting approaches to prevent hyperexcitability related to neuronal death. The recently cloned T-type α_{1G} channel (Perez-Reyes et al., 1998) is expressed mainly in the brain, whereas α_{1H} channel (Cribbs et al., 1998) is distributed ubiquitously (liver, kidney, etc.).

4.2.2. Inhibition of Ca^{2+} influx into postsynaptic neurons

It has been expected by many investigators that Ca^{2+} channel antagonists suppress Ca^{2+} overload observed during ischemia (Uematsu et al., 1988). However, Ca^{2+} channel antagonists targeted on Ca^{2+} overload may be ineffective due to the following reasons. First, it is already late to administer drugs after onset of stroke, because Ca^{2+} overload by NMDA receptors and/or VDCCs occurs right at the onset of strokes. Second, excessive Ca^{2+} influx through VDCC during ischemia may make only marginal contribution to induction of neuronal death. Since the NMDA receptor channels and VDCCs, that constitute the major pathways for excessive Ca^{2+} influx, are different in distribution and localization of increase in $[Ca^{2+}]_i$, their contributions to neuronal death are different between the two pathways. In cultured spinal cord neurons, activation of NMDA receptor resulted in cell death more extensively than that of VDCCs, when NMDA receptors and VDCCs were activated so that they lead to equivalent initial increase in $[Ca^{2+}]_i$, (Tymianski et al., 1993).

When neurons resume their activity after reperfusion, Ca^{2+} influx through VDCCs may play an important role in pathological development of ischemic neuronal death. In addition to immediate damages, Ca^{2+} influx through VDCCs may cause gene expressions through activation of calmodulin kinases, type I adenylate cyclase or Ras protein (reviewed by Ghosh and Greenberg, 1995). Induced gene expression may promote delayed neuronal death via apoptosis. An attractive hypothesis is that Ca^{2+} channel antagonists are able to prevent the progressing apoptotic cascades. In fact, blockers for L-types suppressed synaptically activated expression of the transcription factor genes, *c-fos*, *jun-B*, *zif268*, and *fos-b*, in cultured cortical neurons (Murphy et al., 1991). However, protective effects of L-type VDCC antagonists is marginal in the in vitro model of ischemia (anoxia) (see Section 5).

In regards to the role of Ca^{2+}, it should be noted that cell death through apoptosis is quite different from necrosis induced by glutamate–Ca^{2+} neurotoxicity. Modest increase in $[Ca^{2+}]_i$ inhibited apoptotic cell death, whereas Ca^{2+} is always toxic in glutamate hypothesis. Furthermore, prolonged exposure to L-type VDCC antagonists can induce apoptosis in cortical neurons (Koh and Cotman, 1992).

5. Neuroprotection by Ca^{2+} channel antagonists

Numerous reports have been made on the neuroprotective effects of Ca^{2+} channel antagonists in anoxia/ischemia. Since duration of the insult, dosing schedule, and age or stage of development of neurons may all be important determining parameters, it is not surprising that different models have yielded somewhat conflicting results in evaluating Ca^{2+} channel antagonists (for example, see Table 2). In general, L-type or non-selective Ca^{2+} channel antagonists appear to be more effective in studies using focal ischemia models rather than global ischemia models (Lipton, 1991).

5.1. L-type VDCC antagonists

Earlier expectation of Ca^{2+} channel antagonists as effective therapeutic agents for stroke derived from the belief that improved blood circulation in brain areas would reverse cellular pathology. The drugs developed were therefore derivatives of the classical Ca^{2+} channel antagonists used for cardiovascular diseases. A dihydropyridine, nimodipine (Fig. 3) was reported for its selectivity to L-type in the cerebral artery and neuroprotection from ischemia in animal models (Steen et al., 1983). The compound is the only Ca^{2+} channel antagonist extensively evaluated in double-blind clinical studies. Although nimodipine administration gave positive results in the treatment of subarachnoid hemorrhage (Toni et al., 1991), effectiveness of the drug in acute ischemic stroke was not as convincing after several studies including a large multicenter study (The American Nimodipine Study Group, 1992).

Nicardipine (Alps et al., 1987), isradipine (Sauter et al., 1989) showed neuroprotection in animal models. However, their clinical benefits are marginal (Yao and Ding, 1990). Nilvadipine attenuated ischemic degradation of cytosckeletal proteins of gerbil brain (Kuwaki et al., 1989). Other

Table 2
New Ca^{2+} channel antagonists in animal models of cerebral ischemia

Drug	Species/model	Dose	Outcome	Reference
NNC09-0026	Gerbil/global, 2VO	30 mg/kg, i.p. at −30 min, +24 h and +48 h after occlusion	↑	O'Neill et al., 1997
NNC09-0026	Gerbil/global, 2VO	30 mg/kg, i.p. at +0, +24 h and +48 h after occlusion	→	O'Neill et al., 1997
NNC09-0026	Gerbil/global, 2VO	30 mg/kg, i.p. at +30 min after occlusion, and 3, 10 or 30 mg/kg at 24 and 48 h post-ischemia	↑	Sheardown et al., 1993
NNC09-0026	Gerbil/global, 2VO	30 mg/kg, i.p. at +30 min after occlusion	→	Sheardown et al., 1993
NNC09-0026	Rat/focal, MCAO	30 mg/kg, i.v. slowly over 1 h, beginning 30 min after occlusion	↑	Barone et al., 1994
CNS-1237	Gerbil/global, 2VO	30 mg/kg, i.p., 30 min before and 2.5 h after occlusion	↑	O'Neill et al., 1997
CNS-1237	Rat/focal, MCAO	3 mg/kg bolus followed by a 4 h infusion of 0.75 mg/kg/h	↑	Goldin et al., 1995
SB201823-A	Gerbil/global, 2VO	10 mg/kg, i.p., 30 min before and 2.5 h after occlusion	→	O'Neill et al., 1997
SB201823-A	Rat/focal, PIT	30 mg/kg, i.p., 10 min after illumination and 10 mg/kg at 1 h post-illumination and then twice daily for 3 days	↑	Benham et al., 1993
SB201823-A	Gerbil/global, 2VO	10 mg/kg, i.p., 30 min after occlusion	↑	Benham et al., 1993
SB201823-A	Rat/mouse/focal, MCAO	10 mg/kg, i.v. (rat) or i.p. (mouse), 30 min after occlusion	↑	Barone et al., 1995
SB206284 A	Gerbil/global, 2VO	30 mg/kg, i.p. at +30 min after occlusion, then at 10 mg/kg twice a day for 3 days	→	Wood et al., 1997
SB206284A	Rat/focal, MCAO	10 mg/kg, i.v. slowly over 1 h, beginning 30 min after occlusion	↑	Wood et al., 1997
SB206284A	Rat/focal, PIT	10 mg/kg, i.v. over 15 min, beginning 10 min after illumination; or 10 mg/kg, i.p., 2 h after illumination and twice daily for 3 days; or 10 mg/kg, i.p., 4 h after illumination and twice daily for 3 days	↑	Wood et al., 1997
NS-649	Gerbil/global, 2VO	50 mg/kg, i.p., 30 min before and 2.5 h after occlusion	→	O'Neill et al., 1997
NS-649	Mouse/focal, MCAO	50 mg/kg, i.p. at +30 min, 6, 24 and 48 h after occlusion	↑	Varming et al., 1996
NS-638	Gerbil/global, 2VO	30 mg/kg, i.p. at 1, 4 and 24 after occlusion	→	Moller et al., 1995
NS-638	Mouse/focal, MCAO	50 mg/kg, i.p. at 1, 6, 24 and 48 h after occlusion	↑	Moller et al., 1995
T-477	Rat/focal, PIT	0.3 mg/kg, i.v. bolus immediately after illumination followed by a 24 h infusion of 0.3 mg/kg/h	↑	Ishii et al., 1996
T-477	Gerbil/global, 2VO	30 mg/kg, i.p., 30 min before occlusion	↑	Okamoto (unpublished data)

2VO, bilateral carotid artery occlusion.
MCAO, middle cerebral artery occlusion.
PIT, photochemically-induced thrombosis.
↑, neuroprotective.
→, no effects.

dihydropyridines, lemdipine (NB-818) (Kamei et al., 1991), S-312-d (*S*-(+)-methyl-4,7-dihydro-3-isobutyl-6-methyl-4-(3-nitrophenyl) thieno [2, 3-b] pyridine-5-carboxylate) (Yasui and Kawasaki, 1994), F-0401 ((+/−)-(*E*)-3-[4-(1-imidazolyl)methylphenyl]-2-propen-1-yl methyl 1,4-dihydro-2, 6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate) (Chen et al., 1994) also demonstrated neuroprotection in whole animal models or in hippocampal slices.

Derivatives of other conventional Ca^{2+} channel antagonists have been synthesized for stroke treatment. A diltiazem analog, clentiazem (Kikkawa et al., 1994) and a verapamil analog, (*S*)-emopamil (levemopamil) (Defeudis, 1989) (Fig. 3) dilated cerebral artery and showed protective effect in the animal model.

All the compounds mentioned above are L-type-selective VDCC antagonists. It is unknown whether their bene-

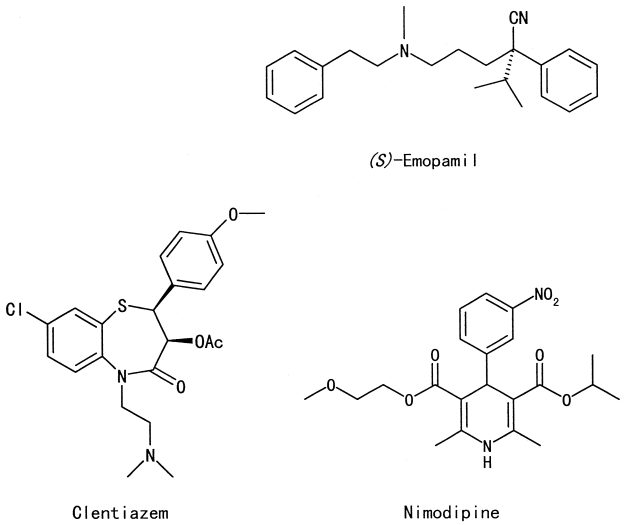

Fig. 3. Chemical structures of L-type Ca^{2+} channel antagonists which show neuroprotective effects in animal models. Dihydropyridine type, nimodipine; phenylalkylamine type, levemopamil; and benzothiazine type, clentiazem.

ficial effects in the animal model are due to improvement of blood circulation or to direct action on neurons. Since some of the VDCC antagonists including nimodipine (Van den Kerckhoff and Drewes, 1985) showed high permeability through the blood–brain barrier, they are capable of blocking L-type VDCCs in neurons (α_{1C}, α_{1D}). However, in the studies using hippocampal slices, nifedipine (Pringle et al., 1996) and nimodipine (Kass et al., 1988) did not show protective effects, suggesting that contribution of blockade of neuronal L-type VDCC to neuroprotection is marginal in the in vitro model. A beneficial effect of nimodipine on hemorrhagic stroke may indirectly derive from its causing hypotension, because the patients, in many cases, suffer from hypertension which facilitates repetitive hemorrhage.

5.2. Non-selective VDCC antagonists

Flunarizine (Fig. 4) showed efficient cerebral protection in animal models (Deshpande and Wieloch, 1986). Although flunarizine is able to improve cerebral circulation, it was reported that the effect was not related to improvement of cerebral circulation (Beck et al., 1988). In fact, the drug showed neuroprotection even in the experiments using hippocampal slices (Amagasa et al., 1990). In contrast, in a double-blind clinical trial, flunarizine did not improve neurologic and functional outcome in patients with acute ischemic stroke (Franke et al., 1996). Flunarizine is a potent non-selective VDCC antagonist. It blocks L- and T-type VDCC in smooth muscle cells (Akaike et al., 1989a), low and high voltage-activated VDCCs in hypothalamus neurons (Akaike et al., 1989b). Flunarizine inhibits most of Ca^{2+} entry into K^+-depolarized cortical synaptosomes and cultured neurons (Kobayashi et al., 1992). The drug interacts with Na^+ channels, and thereby blocks Na^+ influx which may also participate in inducing ischemic neuronal damage (Pauwels et al., 1989). Drugs such as flunarizine that exert multiple actions may block the cascades of neuronal death more efficiently than drugs with a single action site (Spedding et al., 1995).

Many flunarizine analogs have been synthesized. Cinnarizine (Poignet et al., 1989) and lifarizine (RS-87476) (Alps et al., 1995) demonstrated neuroprotection from ischemia in animal models. Lomerizine (KB2796) exerted protective effects not only in the conventional ischemic model (Yamashita et al., 1993), but also in chronic atrophy. One month after transient focal ischemia, the compounds showed beneficial effects on atrophy in non-

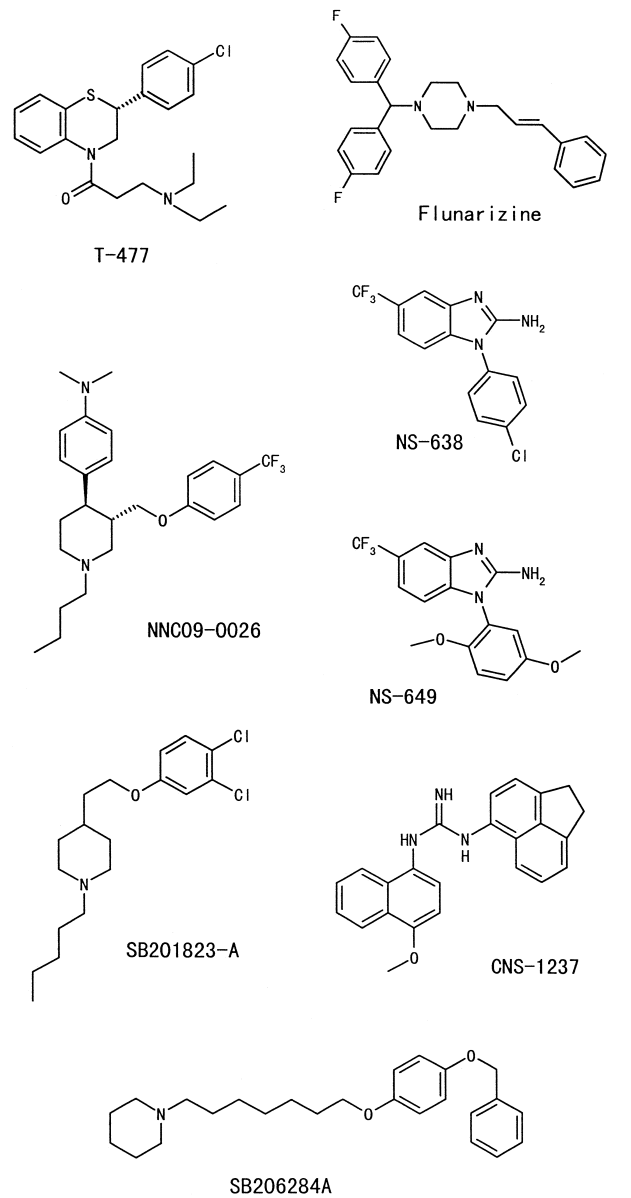

Fig. 4. Chemical structures of non-selective Ca^{2+} channel antagonists which show neuroprotective effects in animal models.

ischemic regions, the substantia nigra and the thalamus, remote from primary ischemic regions, the cortex and striatum (Hara et al., 1993). Lomerizine and flunarizine attenuated the expression of c-Fos-like immunoreactivity, an indicator of neuronal activation, after KCl-induced cortical spreading depression (Shimazawa et al., 1995). Another flunarizine analog, NC1100 ((+/−)-1-(3,4-dimethoxyphenyl)-2-(4-diphenylmethylpiperazinyl)ethanol), attenuated ischemic and postischemic damages to brain metabolism in spontaneously hypertensive rats (Sadoshima et al., 1992).

Neuroprotective action was also tested for the non-selective Ca^{2+} channel antagonists other than flunarizine derivatives (Table 2; Fig. 4). SB 201823-A (4-[2-(3,4-dichlorophenoxy) ethyl]-1-pentyl piperidine hydrochloride), administered 30 min after focal ischemia, showed neuroprotection without affecting blood pressure (Benham et al., 1993; Barone et al., 1995). The compound blocked Ca^{2+} currents of neurons with little selectivity among VDCC subtypes (IC_{50}s ~ 5 μM). It also blocked Na^+ and K^+ currents with lower affinity (IC_{50}s ~ 20 μM). Another compound SB 206284-A (1-[7-(4-benzyloxyphenoxy)heptyl] piperidine) also blocked neuronal high voltage-activated Ca^{2+} channels with little subtype selectivity. SB 206284-A reduced lesion volume in rat photothrombotic model even when the first dose was delayed up to 2–4 h after surgery (Wood et al., 1997). The effects of SB 206284-A on blood pressure and heart rate were insignificant, gaining an advantage over SB 201823-A that produces significant bradycardia.

NNC 09-0026 ((−)-*trans*-1-butyl-4-(4-dimethylaminophenyl)-3-[(4-trifluoromethyl-phenoxy)methyl] piperidine dihydrochloride) reduced infarct size in rat middle cerebral artery occlusion model (Barone et al., 1994). Although NNC 09-0026 showed weak cardiovascular effects, its blocking action on Ca^{2+} current was not selective for particular VDCC types. NS-638 (2-amino-1-(4-chlorobenzyl)-5-trifluoromethyl benzimidazole) exerted inhibitory effects on N- and L-type VDCCs and reduced focal stroke injury in mice (Moller et al., 1995). NS-649 (2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benzimidazole), which inhibited Ca^{2+} currents in chick dorsal root ganglion cells and cerebellar granule neurons without type selectivity, displayed protective effects in the mouse middle cerebral artery occlusion model (Varming et al., 1996). CNS-1237 (*N*-acenaphthyl-*N*′-4-methoxynaphth-1-yl guanidine) produced a reduction in infarct volume in the rat focal stroke model of middle cerebral artery occlusion (Goldin et al., 1995). Similar to flunarizine and SB 201823-A, NNC 09-0026, NS-649 and CNS-1237 inhibited VDCC with little channel selectivity and blocked Na^+ channel (O'Neill et al., 1997).

A diltiazem analog, T-477, showed protective activity in the rat photochemically-induced stroke model (Ishii et al., 1996). While the mother compound diltiazem, which selectively blocks L-type, does not show neuroprotection (Wauquier et al., 1985), T-477, which has little selectivity among VDCC types (Kobayashi et al., 1997), showed neuroprotection. As mentioned in the case of flunarizine, neuroprotection becomes more efficient, if other types of channels are blocked together with L-type.

5.3. Neuronal VDCC-selective antagonists

5.3.1. N-type

ω-Conotoxin MVIIA (SNX-111) is a N-type-selective, 25 amino acids peptide blocker (Fig. 5) that demonstrates excellent in vivo efficacy in the focal and global ischemia models (Valentino et al., 1993; Buchan et al., 1994). The compound was effective in preventing damage in rat hippocampal CA1 neurons after transient global ischemia in the rat even though the drug was administered 24 h after ischemic insult. This result suggests that in the first 24 h after ischemia, a pathophysiological pathway to neuronal death can be reversed. It was reported that the Ca^{2+} content in CA1 neurons after the end of 10 min of forebrain ischemia did not differ from that of normal controls until 24 h of recirculation, and increased significantly 48 h after ischemia (Deshpande et al., 1987). Therefore, ω-conotoxin MVIIA exerted neuroprotection *not* by inhibiting massive calcium accumulation occurred during this early period. In addition, ω-conotoxin MVIIA, a very poor blocker of glutamate release, was effective, whereas SNX-230 (ω-conotoxin MVIIC), a highly potent blocker of glutamate release, was ineffective as a neuroprotective agent (Valentino et al., 1993). These findings imply that the mechanism underlying neuronal damage is more complicated than that proposed in the glutamate-toxicity hypothesis.

The availability of compounds such as ω-conotoxin MVIIA and ω-conotoxin MVIIC should aid elucidating the mechanisms that underly ischemic damage and neuropro-

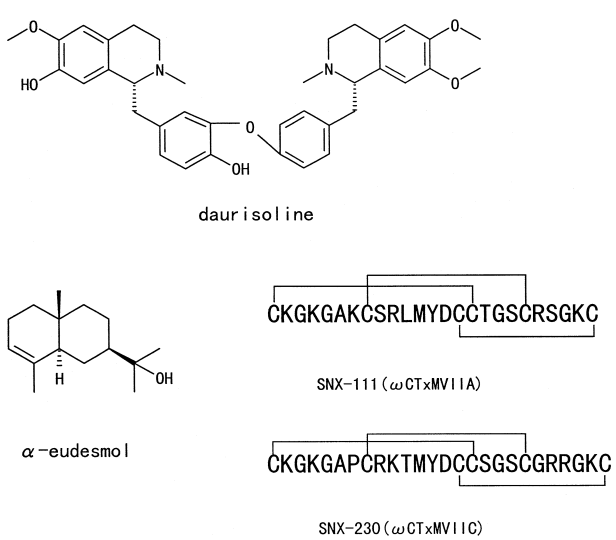

Fig. 5. Chemical structures of selective neuronal Ca^{2+} channel antagonists.

tection. However, there are not many compounds selective to certain neuronal non-L-types. Furthermore, because of low permeability of the peptide across the blood–brain barrier, only 0.03% of ω-conotoxin MVIIA is distributed in the brain (Valentino et al., 1993), which limits clinical utility. Invention of non-peptide blockers permeable across the blood–brain barrier is now essential in both clinical treatments and in basic studies of ischemic neuronal death.

5.3.2. P-type

ω-Agatoxin IVA exhibited a neuroprotective effect in rat hippocampal slices subjected to an in vitro hypoxic–hypoglycemic insult (Small et al., 1995). The neuroprotection activity of ω-agatoxin IVA may be due to the block of Q-type channels, since high concentrations of ω-agatoxin IVA were necessary to exert neuroprotection.

ω-Conotoxin MVIIC, which blocks P- and Q-type VDCCs in addition to N-type, also demonstrated protection against hypoxic neurodegeneration in hippocampal-slice cultures, as well as ω-conotoxin MVIIA (Pringle et al., 1996). However, as indicated above, ω-conotoxin MVIIC was ineffective in the in vivo model (Valentino et al., 1993). This discrepancy may suggest that potent inhibition of glutamate release by ω-conotoxin MVIIC results in neurotoxicity, which mask its own potentiality of neuroprotective action in the whole animal model.

A natural product, eudesmol (Fig. 5), blocked Ca^{2+}-dependent glutamate release from rat synaptosomes, and reduced both brain edema and infarct size in rat middle cerebral artery occlusion model (Asakura et al., 1997). Preliminary studies suggest that eudesmol has blockade selectivity to P-type VDCC.

An alkaloid daurisoline (Fig. 5) blocks P-type Ca^{2+} currents in Purkinje cells (Lu et al., 1994). It did not show significant neuroprotection in rat hippocampal slices after hypoxic/hypoglycemic insult nor in the rat middle cerebral artery occlusion model (Lingenhohl et al., 1997). Daurisoline suppresses respiration, which can be explained by the fact that synaptic transmission at the neuromuscular junction is mediated by P/Q-type Ca^{2+} channels (Protti and Uchitel, 1993; Ousley and Froehner, 1994). This side effect may counteract the beneficial effects in brain ischemia, similar to ω-conotoxin MVIIC.

5.3.3. Other types

Specific antagonists for R and neuronal L-types have yet to be discovered. Specific ligands are needed to elucidate the functions of channels and their involvement in ischemic neuronal death.

T-type Ca^{2+} channels have been suggested to play significant roles physiologically and pathologically. However, since studies of the selective blocker mibefradil (Mishra and Hermsmeyer, 1994) have been focused so far on cardioivascular disease (Clozel et al., 1997), its neuroprotective effects in cerebral ischemia have not yet been reported.

5.4. Discrepancy between valuations set on drugs using animal models and clinical trial

Many compounds which showed potency in the animal models were disappointing in clinical trials. We have to carefully take this discrepancy into consideration in developing drugs. In animal models, experimental conditions are designed so that effective drugs can be systematically differentiated from the ineffective ones. In other words, animal models are already optimized to demonstrate effectiveness of drugs. In clinical trials, on the other hand, large scale, double-blind tests are usually not conducted so frequently. Furthermore, severity of pathological condition and time of drug administration after stroke onset, which all tend to fluctuate, affect the efficacy of the drug. It is difficult to extract the effectiveness of drugs from the highly heterogenous and size-limited sample of patients.

Hunter et al. (1995) discussed problems of using animal models for the study of ischemic stroke. Differences in dosing schedules exist between experimental and clinical investigations. Clinically relevant therapeutic agents must be efficacious when given at least several hours after the insult. In experimental studies, drugs were administered before and/or during, or soon after the ischemic insult, which ensures the penetration of compounds into pathologic areas. Furthermore, pretreatment with drugs may alter cerebral blood flow, plasma glucose levels, cerebral oxygen utilization, or temperature of the animals, which significantly hinder the severity of ischemic damage. Only if the compounds are developed as prophylactics, the dosing schedules in animal experiments become reasonable. Thus, experimental studies are far from representing realistic models of clinical situation.

Assessing criteria of damages are different between animal models and clinical trials. While human studies seek endpoints such as mortality and functional status (especially motor and cognitive performance) of patients following up for weeks and months after stroke, animal studies are usually carried out with short-term follow-ups, hours or a few days. Animal experiments monitor histological (hippocampal CA1 neurons, infarct volume), neurochemical (neurotransmitters, ATP, ions), and hemodynamic (cerebral blood flow) changes, but only infrequently evaluate behavioral/neurological criteria.

6. Concluding remarks

Great efforts have been dedicated to application of Ca^{2+} channel antagonists for the treatment of acute stroke for a long time. Although the researchers were able to show beneficial effects of certain Ca^{2+} channel antagonists in animal models, few to no compound has been proven effective in clinical trials.

It must be stressed that most of the compounds examined so far are derivatives of conventional Ca^{2+} channel antagonists, which are similar to each other in functional characteristics. Some of them are L-type blockers originally developed as vasodilators, and the others displayed no type selectivity. The investigations about 'Ca^{2+} channel antagonists and neuroprotection' were narrowly focused and were not extensive enough to discover Ca^{2+} channel antagonists with efficient neuroprotection activity. However, the finding that neurons are not very vulnerable and are able to survive for several hours, when the blood flow is restored by tissue plasminogen activator, encourages researchers developing novel types of neuroprotective agents. A group of compounds which selectively inhibits each type of neuronal VDCCs may show clinical relevance, and may disclose the mechanisms underlying development of ischemic neurodegeneration as well.

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