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Nicergoline inhibits T-type Ca^{2+} channels in rat isolated hippocampal CA1 pyramidal neurones

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1 The effects of nicergoline on the T- and L-type Ca^{2+} currents in pyramidal cells freshly isolated from rat hippocampal CA1 region were investigated by use of a 'concentration-clamp' technique. The technique combines a suction-pipette technique, which allows intracellular perfusion under a single-electrode voltage-clamp, and rapid exchange of extracellular solution within 2 ms.

2 T-type Ca^{2+} currents were evoked by step depolarizations from a holding potential of −100 mV to potentials more positive than −70 to −60 mV, and reached a peak at about −30 mV in the current-voltage relationship. Activation and inactivation of T-type Ca^{2+} currents were highly potential-dependent.

3 Nicergoline and other Ca^{2+} antagonists dose-dependently blocked the T-type Ca^{2+} channel with an order of potency nicardipine > nicergoline > diltiazem.

4 The L-type Ca^{2+} channel was also blocked in the order nicardipine > nicergoline > diltiazem, although the T-type Ca^{2+} channel was more sensitive to nicergoline.

5 The inhibitory effects of nicergoline and nicardipine on the T-type Ca^{2+} current were voltage-, time-, and use-dependent, and the inhibition increased with a decrease in the external Ca^{2+} concentration. Diltiazem showed only a use-dependent block.

Introduction

Recent evidence suggests that hypoxic-ischaemic neuronal damage could be mainly related to glutamate neurotoxicity and the lethal Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Choi, 1988). In the rat and mongolian gerbil models of vessel occlusion, treatment with nicardipine protected against 'delayed neuronal death' and ischaemia of the hippocampal CA1 pyramidal cells and the cerebellar Purkinje cells (Alps *et al.*, 1986; 1987). Flunarizine also reduced the ischaemic damage by directly acting on the brain tissue rather than on the increase in blood flow in hippocampal areas (Beck *et al.*, 1988). Neurones have three types of voltage-dependent Ca^{2+} channel (T-, N- and L-type Ca^{2+} channels) (Tsien *et al.*, 1988). Interestingly, flunarizine suppressed more strongly T-type Ca^{2+} channels than L-type channels in the rat hypothalamic neurones (Akaike *et al.*, 1989a). These results suggest that part of the ameliorative effect of flunarizine in ischaemic brain damage may be due to the blocking of Ca^{2+} entry to CNS neurones through the T-type Ca^{2+} channel.

Nicergoline, an ergot alkaloid derivative (Figure 2a), which has α_1-adrenoceptor blocking and calcium antagonistic properties (Heitz *et al.*, 1986), protects against ischaemic brain damage induced by bilateral carotid arterial ligation in the ICR-strain of mice and mongolian gerbils (Shintomi *et al.*, 1986). The mechanism of this protective effect is not known. In the present experiments, we examined the effect of nicergoline on the T-type Ca^{2+} channel in pyramidal neurones freshly isolated from the rat hippocampal CA1 region (these neurones are known to be highly sensitive to ischaemia) by using a 'concentration-clamp' technique (Akaike *et al.*, 1986). The mode of action of nicergoline was compared with those of two organic Ca^{2+} antagonists, nicardipine and diltiazem.

Methods

Preparation

Dissociated hippocampal CA1 pyramidal neurones were obtained from 7 to 15 day-old rats, as described previously (Takahashi *et al.*, 1989a). In brief, the brain was removed from rats anaesthetized with ether and dissected into slices (about 500 μm thick), with a microslicer (D.S.K., model DTK-1000). The slices containing the hippocampal region were selected and preincubated in an incubation solution bubbled well with 95% O_2 and 5% CO_2 for 50 min at 31°C. After preincubation they were enzymatically treated in an incubation solution with 0.25 mg ml^{-1} pronase for 20 min, and successively with 0.25 mg ml^{-1} thermolysin for another 20 min. After the enzyme treatments, the slices were washed at least 3 times with a Ca^{2+}-free-EGTA incubation solution. Then, the hippocampal CA1 region was punched out and dispersed mechanically by pipetting. Isolated brain cells were kept in a normal external solution containing 1% bovine serum at room temperature (20°C to 22°C). They were viable for electrophysiological studies for up to 15 h.

Selected neurones were transferred to a culture dish (Corning, 35 mm) and drawn into the opening of a glass pipette (1.5–1.7 μm in diameter) filled with the internal solution described below. The membrane patch in the opening was destroyed by adding negative pressure, and soluble cell contents were exchanged with the pipette-filling solution by diffusion (Akaike *et al.*, 1989a).

Electrical measurement

Transmembrane currents were recorded by a voltage clamp amplifier (Nihon Kohden, type CEZ-2200), monitored on an oscilloscope (Toshiba, type 10M63) and stored on an FM tape recorder (TEAC, type MR-30) for computer data analysis (NEC, type PC-98XL$_2$).

The amplitude of the inward current was measured at the peak of each current. The linear components of the transient capacitative and leakage currents associated with the ionic currents were subtracted during the experiments, by adding the current response to equal but opposite voltage steps using a signal averager (Nihon Kohden, ATAC-150).

Solutions

The incubation solution contained (in mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26 and glucose 10. Ca^{2+}-free-EGTA incubation solution contained (in mM):

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NaCl 124, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 26, glucose 10 and EGTA 2. Normal external solution contained (in mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10 and HEPES 10. The pH was adjusted to 7.4 by adding Tris-base. To separate voltage-dependent Ca^{2+} currents from other Na^+ and K^+ currents, we used Na^+- and K^+-free external solution containing 10 mM Ca^{2+}. The composition was (in mM): choline-Cl 140, CsCl 5, $CaCl_2$ 10, glucose 10 and HEPES 10. The pH was adjusted to 7.4 with Tris-base. The internal solution (pipette solution) was (in mM): N-methyl-D-glucamine fluoride (NMG-F) 100, tetraethylammonium chloride (TEA-Cl) 20 and HEPES 10. The pH was adjusted to 7.2 with Tris-base. To record L-type Ca^{2+} currents, we used an internal solution containing NMG-aspartate 100 mM, CsCl 30, TEA-Cl 20, ATP-Mg 5, HEPES 10 and EGTA-Ca buffer (free $Ca^{2+} = 10^{-8}$ M). The pH was adjusted to 7.2 by adding Tris base. All experiments were carried out at room temperature (20–22°C).

Drugs

Drugs used in the experiments were: pronase (Hoechst), thermolysin (Sigma), N-methyl-D-glucamine (Tokyo Kasei), nicergoline and diltiazem (Tanabe) and nicardipine (Yamanouchi). All drugs were applied to the isolated neurones by an extremely rapid concentration jump termed the 'concentration-clamp' method, which combines the suction-pipette technique for intracellular perfusion (Akaike *et al.*, 1978) and a technique for the exchange of external solution within 2 ms (see Figure 1 in Akaike *et al.*, 1986). Organic Ca^{2+} antagonists were examined in a dark room.

Results

Separation of T-type Ca^{2+} currents in hippocampal neurones

Four types of extracellular Ca^{2+}-sensitive and voltage-dependent inward currents were observed in rat isolated hippocampal CA1 pyramidal neurones bathed with Na^+- and K^+-free external solution containing 10 mM Ca^{2+}: a rapid transient current (tetrodotoxin-sensitive current), a transient current (T-type or low-threshold Ca^{2+} current), a slowly-inactivated current (N-type Ca^{2+} current), and a sustained-steady current (L-type or high threshold Ca^{2+} current) (Takahashi *et al.*, 1989b). To isolate T-type Ca^{2+} currents from other types of Ca^{2+} currents, the rapid transient current was blocked first by adding tetrodotoxin (TTX) 10^{-7} M (a concentration which completely blocks the voltage-dependent Na^+ channels, Kaneda *et al.*, 1989). Then, the L-type Ca^{2+} current was suppressed irreversibly by intracellular dialysis with F^- for 5–15 min, as observed in other preparations (Akaike *et al.*, 1983; Carbone & Lux, 1987). Since T-type Ca^{2+} currents could only be separated from the N-type by kinetic curve fitting of the current inactivation, we selected neurones which do not contain N-type Ca^{2+} current component.

T-type Ca^{2+} channels of hippocampal neurones could be activated by a 300 ms step depolarization to about −70 to −60 mV from a holding potential (V_H) of −100 mV in the presence of 10^{-7} M TTX. The current amplitude increased with increasing depolarization, reaching a peak at about −30 mV (Figure 1). The activation and inactivation processes of the T-type Ca^{2+} channel were expressed as a single exponential function and were highly dependent on potential. The kinetic characteristics of T-type Ca^{2+} currents were identical to those found in chick and rat sensory neurones (Carbone & Lux, 1984), in rat hypothalamic neurones (Akaike *et al.*, 1989a) and also in amygdaloid neurones (Kaneda & Akaike, 1989).

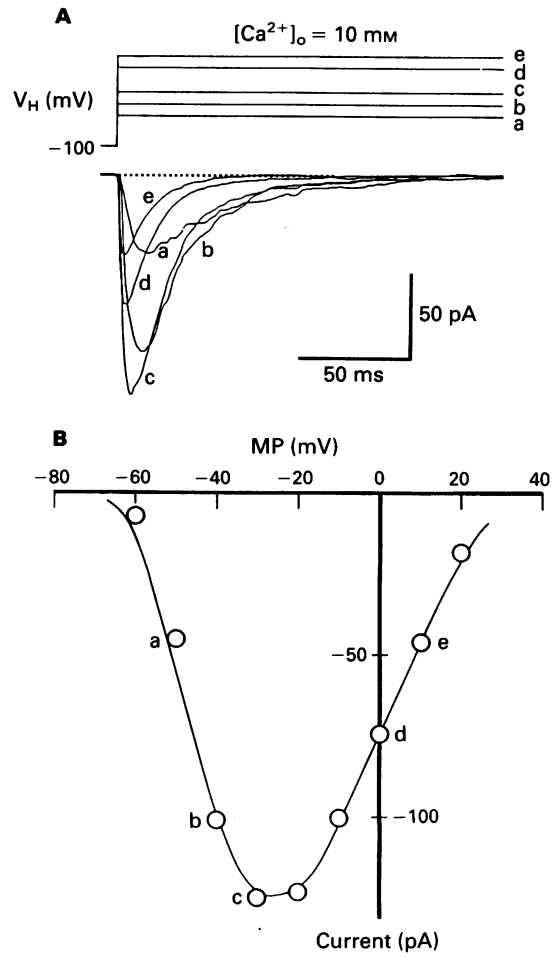

Figure 1 Low threshold or T-type Ca^{2+} current ($T\text{-}I_{Ca}$) in rat isolated hippocampal CA1 neurone. (A) A typical example of $T\text{-}I_{Ca}$ recorded from a neurone immersed in the external solution containing 10 mM Ca^{2+} (see Methods). Currents were induced by depolarizing pulses to various potentials (a, −50; b, −40, c, −30; d, 0; e, +10 mV) from a holding potential (V_H) of −100 mV. (B) The current voltage (I-V) relationship $T\text{-}I_{Ca}$ from the same neurone. MP, membrane potential (mV).

Effect of nicergoline on T-type Ca^{2+} currents

The experiments were performed on isolated hippocampal neurones immersed in the external solution containing 10 mM Ca^{2+}. The effect of nicergoline was examined on T-type Ca^{2+} currents induced by a 300 ms step depolarization to −20 mV from a V_H of −100 mV every 30 s. The inhibitory action of nicergoline reached a steady-state level within 30 s of the application of the drug. The inhibition was completely reversible (Figure 2b). A similar time-dependent inhibition was observed on addition of diltiazem. The inhibition by nicardipine of the T-type Ca^{2+} current developed much more slowly compared with those of nicergoline and diltiazem. Therefore, the measurements were made exactly 2 min after the respective concentrations of these drugs had been added. As shown in Figure 3, the order of blocking efficacy was nicardipine > nicergoline > diltiazem. The concentrations which gave half the maximum inhibition (IC_{50}) were 1.75×10^{-6} M for nicardipine, 1.4×10^{-5} M for nicergoline and 2.1×10^{-4} M for diltiazem.

Voltage- and time-dependent inhibitory actions of nicergoline

The voltage-dependence of the inhibitory action of nicergoline and the other two Ca^{2+} antagonists on the T-type Ca^{2+} channel was studied by following the conventional double

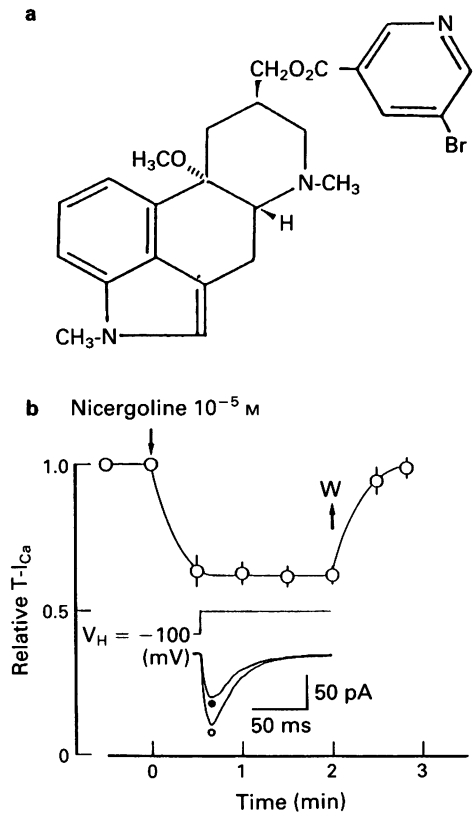

Figure 2 (a) Chemical structure of nicergoline ($C_{24}H_{26}BrN_3O_3$; molecular weight 484.39). (b) Time course of the inhibitory effect of nicergoline on a T-type Ca^{2+} current (T-I_{Ca}). The drug application was made at the 'arrow' above the graph. 'W' indicates the washout of the drug. Extracellular Ca^{2+} concentration ($[Ca^{2+}]_0$) was 10 mM. Inset shows typical T-I_{Ca} evoked by a 300 ms depolarizing potential from a V_H of −100 mV to −20 mV with (●) or without (○) 10^{-5} M nicergoline. Each point is the mean of 4 neurones and vertical lines show s.e.mean.

pulse methods: (1) A prepulse (PP) lasting for 3 s at various membrane potentials between −120 and −40 mV was first applied followed by a test pulse (TP) from −100 to −20 mV of 300 ms duration, which was sufficient to induce maximum inward current. The interval between PP 'off' and TP 'on' was set to 2 ms. The individual insets of Figure 4 show the relationships between steady-state inactivation and membrane potential (h_∞-Vm curve), with or without nicergoline and other Ca^{2+} antagonists. Both nicergoline and nicardipine shifted the respective h_∞-Vm curves toward the hyperpolarizing direction, thereby indicating that these compounds had a voltage-dependent inhibitory action. Diltiazem, on the other hand, did not shift the h_∞-Vm curve.

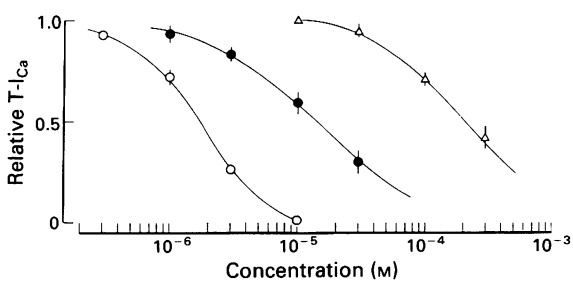

Figure 3 Effects of nicergoline (●), nicardipine (○) and diltiazem (△) on T-type Ca^{2+} currents (T-I_{Ca}). The currents were evoked by a 300 ms step depolarization to −20 mV from a V_H of −100 mV every 30 s. $[Ca^{2+}]_0$ = 10 mM. Each point is the mean of 4–5 neurones and vertical lines show s.e.mean.

(2) PPs of various durations (10 ms to 10 s) were also applied, which induced half-maximum steady-state inactivation ($h_{0.5}$) with a PP of 3 s, followed by a TP lasting for 300 ms at −20 mV. In these experiments, the relative amplitude of the T-type Ca^{2+} current as a function of PP duration was much smaller in the presence of both nicergoline and nicardipine. The current inhibition by these drugs was enhanced by increasing PP duration (Figure 4). The results indicate that both nicergoline and nicardipine exert a time-dependent inhibitory effect. On the other hand, the inhibitory effect of diltiazem was not affected by changing the duration of the PP (Figure 4).

Use-dependent effect of nicergoline

To test the possible use- or frequency-dependent inhibition of nicergoline and other Ca^{2+} antagonists on T-type Ca^{2+} channels, trains of command pulses (−100 to −20 mV for 300 ms) were applied at different pulse intervals ranging from 1 to 30 s. In the absence of drugs, the T-type Ca^{2+} current remained fairly constant up to a 5 s interval. When pulse intervals shorter than 1 s were applied, the inhibition of the current increased with each stimulus and the block was cumulative (Figure 5a). In the presence of nicergoline (10^{-5} M) and diltiazem (10^{-4} M), no measureable use-dependent effect was observed when the pulse interval was shorter than 5 s, although considerable tonic inhibition of the T-type Ca^{2+} current occurred when the stimulation was applied every 1 s. After addition of nicardipine (3×10^{-6} M), an additional decrease of Ca^{2+} current was observed even at stimulation intervals of 5 s; the inhibition increased with each pulse and the block was also cumulative.

Since the effect of Ca^{2+}-antagonist on the T- and L-type Ca^{2+} channels in neurones and cardiac cells depends on the extracellular Ca^{2+} concentration ($[Ca^{2+}]_0$) (Akaike *et al.*, 1989a,b; Lee & Tsien, 1983; Takahashi & Akaike, unpublished observation), we also examined the blocking potency of nicergoline and nicardipine on T-type Ca^{2+} channels in neurones immersed in an external solution containing 2.5 mM Ca^{2+}. In these experiments, the T-type Ca^{2+} current was evoked by a pulse interval of 1 s. The results are summarized in Figure 6, in which the inhibition by nicergoline and nicardipine of T-type Ca^{2+} currents was much facilitated as compared with that in the external solution containing 10 mM Ca^{2+}. The IC_{50} value was 6.2×10^{-7} M for nicergoline and 1.4×10^{-7} M for nicardipine.

Effect of nicergoline on L-type Ca^{2+} currents

The effect of nicergoline was examined on the L-type Ca^{2+} current induced by step depolarization to +10 mV from a V_H of −50 mV; at this holding potential the T-type Ca^{2+} channel was completely inactivated and only the L-type Ca^{2+} channel was recorded. The effect of each drug was measured after a steady-state had been achieved after its application. In the external solution containing 10 mM Ca^{2+}, each drug inhibited the L-type Ca^{2+} current in a dose-dependent manner and the values for IC_{50} were 3.8×10^{-6} M for nicardipine, 1.75×10^{-5} M for nicergoline, and 1.06×10^{-4} M for diltiazem (n = 5–8).

Discussion

In rat isolated hypothalamic and hippocampal neurones, nicardipine dose-dependently blocked T-type Ca^{2+} channels (Akaike *et al.*, 1989a; Takahashi *et al.*, 1989b). In the present study in rat isolated hippocampal neurones, nicergoline showed voltage-, time- and use-dependent inhibition of the T-type Ca^{2+} channel (Figures 4 and 5). The blocking potency of nicergoline on T-type Ca^{2+} channels was weaker than that of nicardipine, but stronger than that of diltiazem.

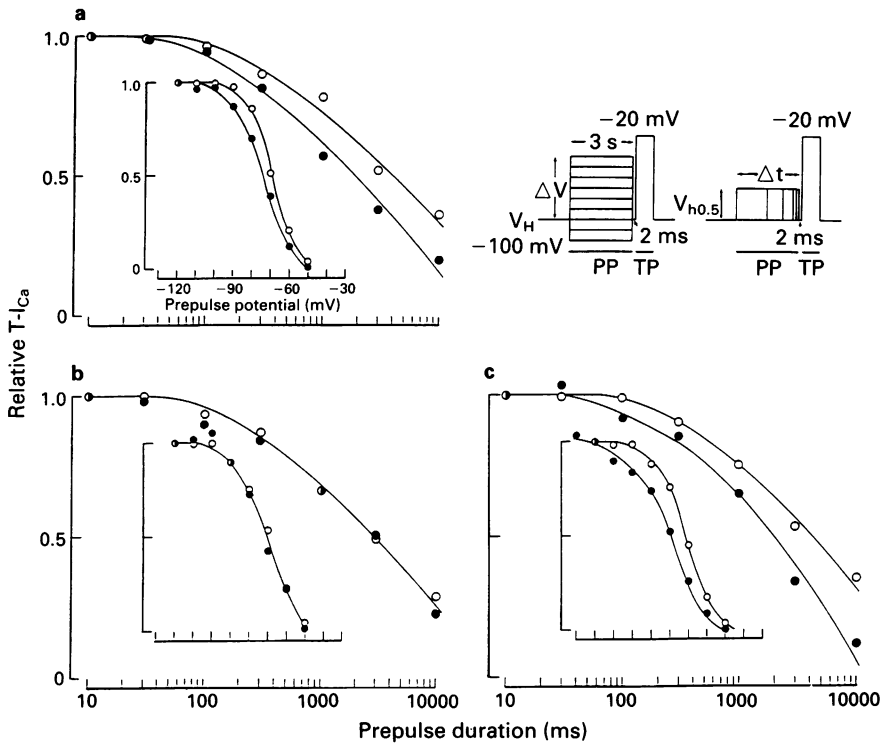

Figure 4 Voltage- and time-dependent inhibitory effects of (a) nicergoline (10^{-5} M), (b) diltiazem (3×10^{-4} M) and (c) nicardipine (3×10^{-6} M) on T-type Ca^{2+} currents (T-I_{Ca}). Protocols for double pulse experiments are shown in the upper right of the figure; PP = prepulse and TP = test pulse. Insets show the inactivation of T-I_{Ca} induced by a 3 s long prepulse just before a testing step depolarization to −20 mV from a V_H of −100 mV with (●) or without (○) Ca^{2+} antagonists. Relative amplitudes of T-I_{Ca} were plotted as a function of prepulse potential. Currents were normalized to that of control evoked by the test pulse without a conditioning pulse. Time-dependent inhibition of Ca^{2+} antagonists on T-I_{Ca} was examined with a prepulse of various durations from 10 ms to 10 s. V_H = −100 mV and $[Ca^{2+}]_0$ = 10 mM. Note the strong voltage- and time-dependent inhibition by nicergoline and nicardipine on T-I_{Ca}. Each point is the mean of 4 neurones.

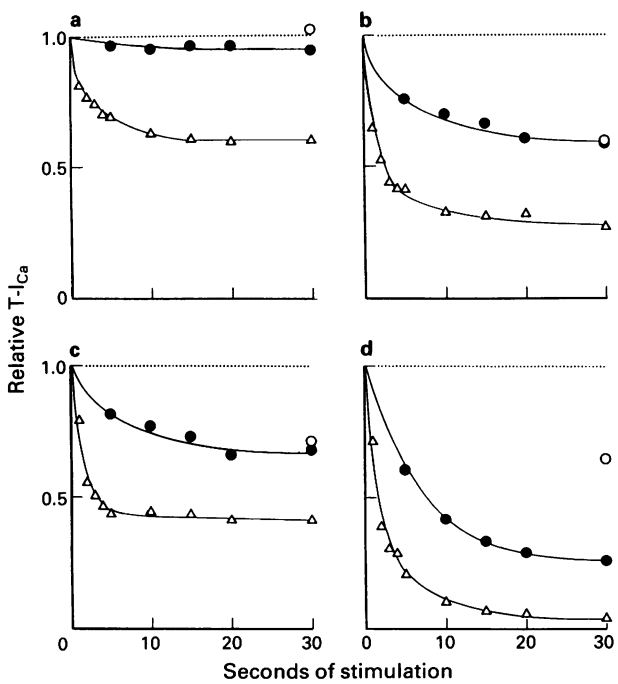

Figure 5 Use-dependent effects of (b) nicergoline (10^{-5} M), (c) diltiazem (10^{-4} M) and (d) nicardipine (3×10^{-6} M) on T-type Ca^{2+} currents (T-I_{Ca}). Trains of depolarizing pulses (−100 to −20 mV for 300 ms) were applied at intervals of 1 (△), 5 (●) and 30 (○) s. Current amplitudes were normalized to that induced by the first test pulse. $[Ca^{2+}]_0$ = 10 mM and V_H = −100 mV. Each point is the mean of 4 neurones. (a) Control.

In rat isolated hypothalamic and hippocampal CA1 pyramidal neurones and rat cultured aorta smooth muscle cells, the inhibitory effects of nimodipine, nicardipine and flunarizine were much stronger on T-type Ca^{2+} channels than on the L-type (Akaike *et al.*, 1989a,b; Takahashi *et al.*, 1989b). On the other hand, diltiazem had the same inhibitory actions on both T- and L-type Ca^{2+} channels (Akaike *et al.*, 1989a). Thus, the present results with nicergoline on T-type Ca^{2+} channels are similar to those obtained with nicardipine and nimodipine in neurones isolated from the CNS.

With the hippocampal neurones used in the present study, an increase in the stimulation rate facilitated the block of the

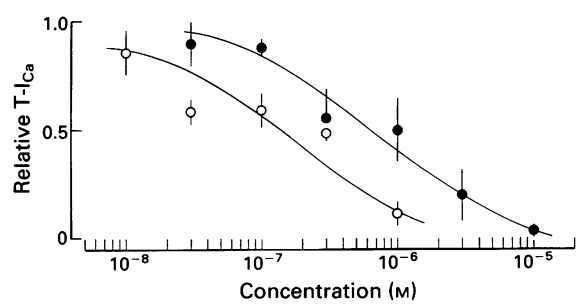

Figure 6 Dose-dependent inhibition by nicergoline (●) and nicardipine (○) of T-type Ca^{2+} currents (T-I_{Ca}) evoked by a 300 ms step depolarization to −20 mV from a V_H of −100 mV every 1 s in an external solution containing 2.5 mM Ca^{2+}. Currents were normalized to that induced by the testing pulse to −20 mV from a V_H of −100 mV without Ca^{2+} antagonists. Each point is the mean of 4–5 neurones and vertical lines show s.e.mean.

T-type Ca^{2+} current by nicergoline and nicardipine, indicating use-dependent block by these drugs. The use-dependent block of organic Ca^{2+} antagonists might be attributed to the difference in the binding and unbinding rates of antagonists at the binding site of the T-type Ca^{2+} channel, which depends on the state of the channel (open, inactivated, resting) (Courtney, 1975). The states associated with membrane depolarization (open and inactivated) showed a greater affinity for nicergoline. Furthermore, the inhibition of the T-type Ca^{2+} current by nicergoline increased time-dependently with long-lasting depolarizing pulses (Figure 4), suggesting that nicergoline, as well as nicardipine, also preferentially binds to inactivated T-type Ca^{2+} channels.

Hypoxic-ischaemic neuronal injury is linked to the excessive inactivation of postsynaptic N-methyl-D-aspartate (NMDA) receptors which may be the predominant route of lethal Ca^{2+} entry (Choi, 1988). This is due to the NMDA-gated channel being highly permeable to both Na^+ and Ca^{2+} (MacDermott *et al.*, 1986), and voltage-gated Ca^{2+} channels such as T-, L-, and N-type Ca^{2+} channels can be activated by NMDA-induced membrane depolarization. According to Choi (1988), since the T-type Ca^{2+} channel has a small conductance and is rapidly inactivated, this channel is unlikely to make a major contribution to the net Ca^{2+} influx, whereas the L-type Ca^{2+} channel with its high conductance and slow inactivation may contribute more to the net Ca^{2+} influxes. However, in the rat hypothalamic neurones (Akaike *et al.*, 1989a) and hippocampal neurones (Takahashi *et al.*, 1989b), flunarizine, nifedipine, nicardipine and nimodipine inhibited both T- and L-type Ca^{2+} channels, and the inhibitory effects of flunarizine and dihydropyridine derivatives were stronger in the T-type Ca^{2+} channel (Akaike *et al.*, 1989a; Takahashi *et al.*, 1989b). In *in vivo* studies, these Ca^{2+} antagonists improved the ischaemic neurological damage in various animals (Alps *et al.*, 1986; 1987; Beck *et al.*, 1988; Hoffmeister *et al.*, 1979; Steen *et al.*, 1983; 1985). Interestingly, when rabbits were subjected to 15 min periods of stagnant cerebral hypoxia, the intravenous administration of phenytoin (an anitconvulsant) afforded significant protection of neurones on the hippocampus and in the dentate nucleus (Cullen *et al.*, 1979). Phenytoin firmly blocks T-type Ca^{2+} channels in rat cultured hippocampal neurones (Yaari *et al.*, 1987) and in freshly isolated hippocampal CA1 pyramidal neurones from the rat (Takahashi *et al.*, 1989b) at a concentration which has little effect on the L-type Ca^{2+} channel. In addition, with respect to the kinetic properties, the T-type Ca^{2+} conductance seems to play an important role by contributing to spontaneous depolarization waves and rebound excitation in the brain. Therefore, these observations suggest that the T-type Ca^{2+} channel is also strongly linked to Ca^{2+}-mediated neurotoxicity.

In the ICR-strain of mice and mongolian gerbils, nicergoline protected against ischaemic brain damage induced by bilateral carotid occlusion (Shintomi *et al.*, 1986). Shintomi *et al.* (1986) proposed that the protective effects of nicergoline on ischaemic brain damage may be due to its ameliorating action on cerebral energy metabolism and partly due to its inhibitory action on lipid peroxide formation. If the possibility is considered that T-type Ca^{2+} channels are linked strongly to Ca^{2+}-mediated neurotoxity and that nicergoline blocks not only the L-type Ca^{2+} current but also the T-type Ca^{2+} current in hippocampal CA1 pyramidal neurones (the present results), one of the protective effects of nicergoline on ischaemic brain damage may be due to its inhibitory actions on T- and L-type Ca^{2+} channels in brain cells.

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