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Inhibitory effects of a new neuroprotective diltiazem analogue, T-477, on cloned brain Ca^{2+} channels expressed in *Xenopus* oocytes

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Abstract

A new neuroprotective agent T-477 ((*R*)-(+)-2-(4-chlorophenyl)-2,3-dihydro-4-diethylaminoacetyl-4H-1,4-benzothiazine) and diltiazem are similar in chemical structures but they show different biological properties. To investigate the properties that differentiate T-477 from diltiazem, we examined the effects of the compounds on a cardiac L-type and brain non-L-type Ca^{2+} channels expressed in *Xenopus* oocytes. Cardiac L-type currents were inhibited by Ca^{2+} channel antagonists with an order of potency: PN200-110 isradipine $\gg$ diltiazem > T-477. Brain BI (class A)-, BII (class E)- and BIII (class B)-type Ca^{2+} channel currents were inhibited by T-477 with an IC_{50} of 45, 74 and 59 μM, respectively, whereas diltiazem barely inhibited the brain non-L-type channels and PN200-110 had no effect. T-477 caused a marked use- and frequency-dependent block of BI Ca^{2+} channel currents, as demonstrated by a cumulative increase of the block during a train of depolarizing pulses, which seemed to be due to a slow repriming of the drug-bound channels from inactivation. These results suggest that T-477 exerts neuroprotection of brain neurons from ischemic neuronal damage through its inhibitory action on brain Ca^{2+} channels that differentiates T-477 from cardiac L-type channel blockers such as diltiazem and PN200-110. © 1997 Elsevier Science B.V.

Keywords: Ca^{2+} channel, cloned; Ca^{2+} channel antagonist; Neuroprotective; T-477 ((*R*)-(+)-2-(4-chlorophenyl)-2,3-dihydro-4-diethylaminoacetyl-4H-1,4-benzothiazine); Oocyte; (*Xenopus*)

1. Introduction

Glutamate neurotoxicity connected to lethal Ca^{2+} influx has been proposed as a major mechanism by which hypoxic–ischemic neuronal damage develops (Choi, 1988). Since both glutamate release and Ca^{2+} influx are controlled by activation of neuronal voltage-dependent Ca^{2+} channels (VDCC), it would be reasonable to expect that blockade of Ca^{2+} influx by VDCC blockers leads to neuroprotection of brain neurons from ischemic damage. In fact, experimental data has already shown that some organic VDCC blockers, Ca^{2+} channel antagonists, are beneficial for early treatment of stroke. Dihydropyridines, nimodipine (Steen et al., 1983) and nicardipine (Alps et al., 1987), and flunarizine (Deshpande and Wieloch, 1986) are effective in the protection of the brain from ischemia in animal models. However, since these antagonists inhibit Ca^{2+} influx in cardiac and smooth muscle cells, thereby showing strong cardiovascular effects, their clinical utility in treatment of stroke is significantly limited. Diltiazem is also a well-known Ca^{2+} channel antagonist vasodilator, but this drug does not show remarkable protective effects in ischemia at all (Wauquier et al., 1985). Until now, there is no compound known for its neuroprotective action through blockade of neuronal VDCC.

Recently, it has been shown that a diltiazem analogue, T-477 ((*R*)-(+)-2-(4-chlorophenyl)-2,3-dihydro-4-diethylaminoacetyl-4H-1,4-benzothiazine, Fig. 1), possesses protective effects on brain neurons in ischemia models in the rats (Ishii et al., 1996), while it exerts only a weak blocking effect on smooth muscle contraction (unpublished results). The inverse relationship between parental diltiazem and its analogue T-477 in two criteria of biological activity, neuroprotection and vasodilation, suggest that the two drugs target distinct action sites, namely, VDCC types

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despite their structural resemblance. Investigating the difference of T-477 in pharmacological properties from diltiazem will provide a great deal of information in structural modification of diltiazem to maximize its neuroprotective activity.

In the present study, the effect of T-477 on the cardiac L-type and non-L-type brain Ca^{2+} channel (BI-α_{1A} (P or Q type)) α_1 subunits expressed together with α_2 and β subunits in *Xenopus* oocytes was examined in comparison with the effects of diltiazem and the dihydropyridine PN200-110 (isradipine). Blocking profiles of T-477 were studied using recombinant BI channels. In order to establish the selectivity of this compound, the agent's ability to block other types of high-voltage activated VDCC was also examined.

2. Materials and methods

cRNAs specific for the cardiac L-type Ca^{2+} channel α_C subunit from human heart (hHT-1, Schultz et al., 1993); the BI-2 (α_{1A}, Mori et al., 1991), BII (α_{1E}, Wakamori et al., 1994), BIII (α_{1B}, Fujita et al., 1993; Wakamori et al., 1994) α_1 subunits from rabbit brain; the cardiac/brain β_{1b} subunit, and the skeletal muscle α_{2a} subunit were synthesized in vitro using cDNAs as templates. *Xenopus* oocytes were co-injected with a total of 50 nl of α_1 subunit cRNA (0.2 μg/μl) in combination with the β_{1b} subunit cRNA (0.2 μg/μl) and α_{2a} subunit cRNA (0.2 μg/μl). The injected oocytes were maintained at 15–20°C in modified Barth's medium (in mM: NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, sodium pyruvate 2.5, theophylline 0.5, pH 7.3 with Tris base) in the presence of penicillin (100 u/ml), streptomycin (100 μg/ml) and gentamicin (50 μg/ml). These cells were studied within 2 to 4 days after injection.

A two-microelectrode voltage clamp method was used with 3 M KCl-filled electrodes (1 to 2 MΩ) connected to a voltage clamp amplifier (Axoclamp 2A, Axon Instruments, Burlingame, CA, USA) to measure whole cell Ba^{2+} currents in oocytes. The pCLAMP software (Axon Instruments) was used for data acquisition and analysis. Currents were digitized at 1 kHz after being filtered at 0.1 kHz. In the experiments of Figs. 1–4, 8 and 9, leakage and capacitive currents were subtracted by the P/4 procedure (Bezanilla and Armstrong, 1977).

The external solution contained (mM): *N*-methyl-D-glucamine 120, KOH 2, $Ba(OH)_2$ 4, and HEPES 5, pH 7.4 with methanesulfonic acid). The experimental chamber (0.3 ml in volume) was perfused continuously with gravity flow at a rate of 2 ml/min. The solution in the chamber was replaced within 30 s. All experiments were performed at room temperature (20–22°C).

T-477 and diltiazem were supplied by Tanabe Seiyaku (Osaka) and Marion–Merrel–Dow (Cincinnati), respectively. PN200-110 was kindly supplied by Dr. R. Hof of Sandoz. The drugs were dissolved in dimethylsulfoxide at 100 mM and diluted to a final concentration with the external solution. Dimethylsulfoxide did not show any effect on currents even at the highest concentration (0.1%).

Experimental values were expressed as means ± S.E.M. Statistical significance was tested by the two-tailed Student's *t*-test.

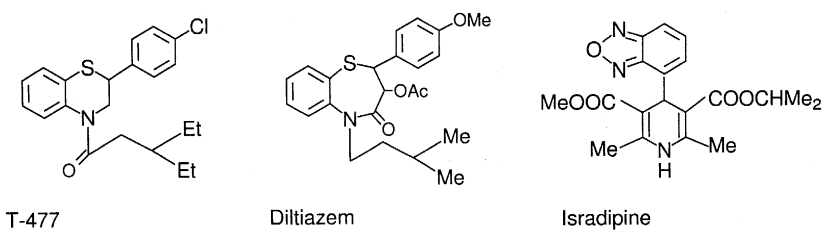
3. Results

3.1. Effects of T-477 and the classical organic Ca^{2+} channel antagonists on cloned cardiac L-type Ca^{2+} channel current

Oocytes expressing the cardiac L-type α_{1C} channel (hHT-1) were immersed in the Na^+- and Cl^--free external solution containing 4 mM Ba^{2+}. The oocytes exhibited inward Ba^{2+} currents through L-type Ca^{2+} channels that showed rapid activation and slow inactivation in response to depolarizing voltage clamp pulses from a holding potential of −80 mV (Fig. 2a). As the magnitude of depolarization was increased, the peak inward current grew larger, reaching the greatest amplitude at about 0 mV (Fig. 2b).

Extracellular application of the benzothiazine T-477 (30 μM) slightly suppressed the L-type α_{1C} channel current, without changing the shape of current–voltage (I–V) relationship curve (Fig. 2 top). Inhibition of the L-type current by the benzothiazepine analogue diltiazem (30 μM) is more prominent than T-477. PN200-110, a dihydropyridine-type Ca^{2+} channel antagonist, showed a similar degree of blockade at a lower concentration (3 μM) (Fig. 2, middle and bottom).

The dose–response relationships for Ca^{2+} channel antagonist blocks of the L-type Ca^{2+} channel current are illustrated in Fig. 3. In this figure, peak current amplitude



T-477 Diltiazem Isradipine

Fig. 1. Chemical structures of T-477, diltiazem and PN200-110 (isradipine).

resulting from a depolarizing test pulse to 0 mV is plotted as a function of drug concentration. The curves in Fig. 3 were drawn using the following equation:

$$I = 1/(1 + [C]/K_d)$$

where I is the relative current amplitude, K_d is the dissociation constant and $[C]$ is the drug concentration. The good fit of this equation indicates that these drugs bind to the channels with a 1:1 stoichiometry. The half-maximal inhibition of the L-type α_{1C} current by T-477 occurred at 52 μM, IC_{50}, equaling the K_d value determined by the equation. As seen in Fig. 2, the inhibition of this channel current by diltiazem and PN200-110 was stronger than that by T-477, with an IC_{50} of 11 and 0.32 μM, respectively.

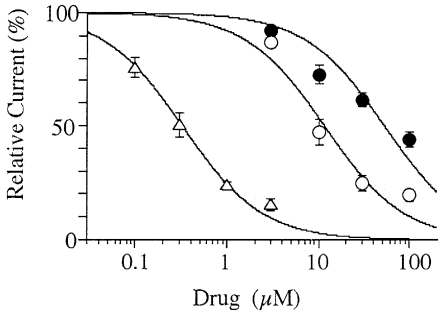


Fig. 3. Dose–response relationship for inhibition of the L-type channel current by T-477 (●), diltiazem (○) and PN200-110 (△). The amplitude of peak current elicited by 400 ms depolarizing pulses of 0 mV from a holding potential of −80 mV at an interval of 15 s was normalized to the control amplitude in the absence of drug. Each point represents the mean ± S.E.M. obtained from 5 experiments.

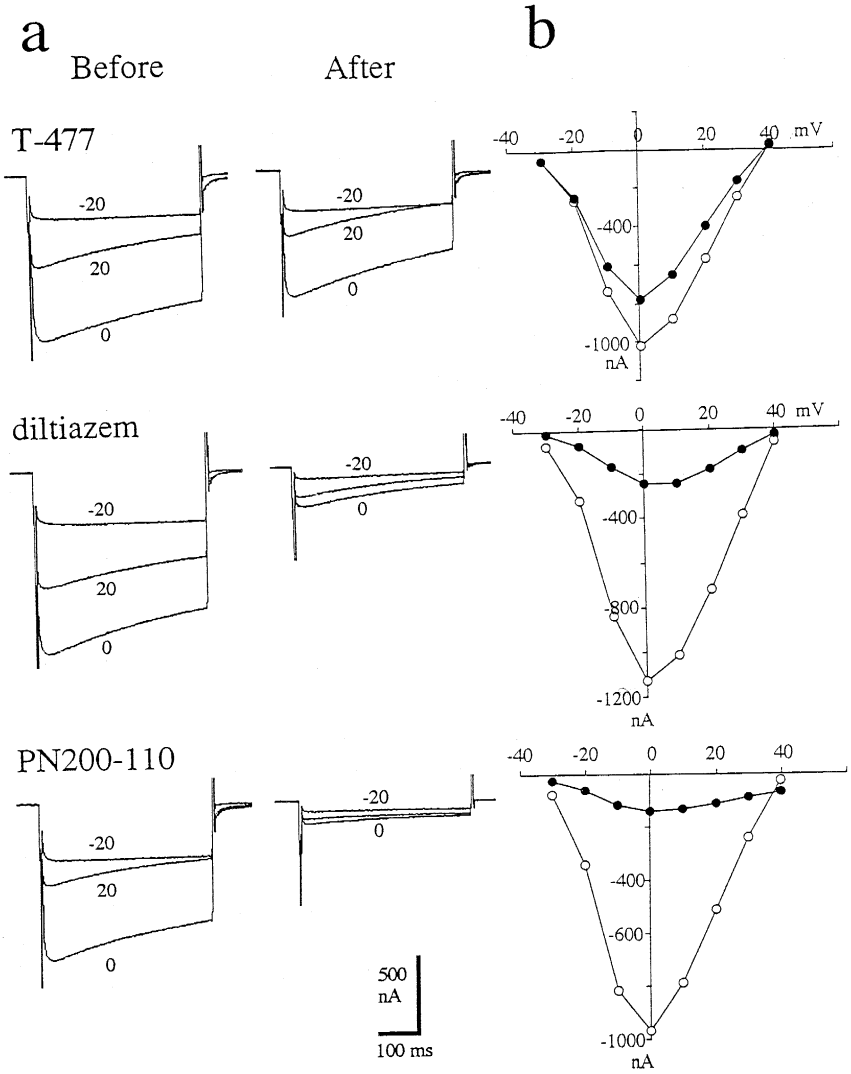


Fig. 2. Effects of Ca^{2+} channel antagonists on recombinant cardiac L-type Ca^{2+} channel currents in *Xenopus* oocytes. (a) Original current traces before (left) and after (right) superfusion with 30 μM of T-477 (top), 30 μM of diltiazem (middle), and 3 μM of PN200-110 (bottom). Currents were elicited by 400 ms depolarizing pulses of −20, 0, 20 mV from a holding potential of −80 mV at an interval of 15 s. (b) The peak current amplitude is plotted as a function of depolarizing step potentials in the absence (○) and the presence (●) of 30 μM of T-477 (top), 30 μM of diltiazem (middle), 3 μM of PN200-110 (bottom).

3.2. Effects of T-477 and the classical organic Ca^{2+} channel antagonists on cloned brain BI Ca^{2+} channel current

The BI α_{1A} subunit generates a ω-agatoxin-IVA-sensitive but dihydropyridine-resistant Ca^{2+} channel current, functionally classified as P- or Q-type (Mori et al., 1991; Sather et al., 1993). In the same external solution used in the measurement of L-type Ca^{2+} channel, the oocytes implanted with BI Ca^{2+} channels exhibited inward Ba^{2+} currents that showed rapid activation and inactivation in response to depolarizing voltage clamp pulses of > -30 mV from a holding potential of -80 mV (Fig. 4a). The peak inward current grew larger with increasing depolarization step, reaching a peak at about 0 mV (Fig. 4b).

Extracellular application of 30 μM T-477 suppressed the BI channel current at all membrane potentials from -20 to 30 mV, without changing the shape of the I–V curve (Fig. 4, top). Compared to T-477, the blocking effect of the BI current by diltiazem at the same concentration (30 μM) was weak (Fig. 4, middle), and inhibition of PN200-110 (30 μM) on the BI current was almost absent (Fig. 4, bottom) (Tang et al., 1993).

The dose–response relationships for blockade of BI currents by the Ca^{2+} channel antagonists at depolarizing pulses to 0 mV (Fig. 5) showed a concentration range of blockade for T-477 similar to that in the L-type currents. The half-maximal inhibition of BI current by T-477 occurred at 45 μM. However, the inhibition of BI channel current by diltiazem and PN200-110 was weak, with an IC_{50} of more than 100 μM, in contrast to that of L-type α_{1C} channel by these two Ca^{2+} channel antagonists (see above).

3.3. Characterization of the inhibitory action of T-477 on BI channel current

Fig. 6 shows the steady-state inactivation curves for BI channels before and after exposure to 30 μM T-477.

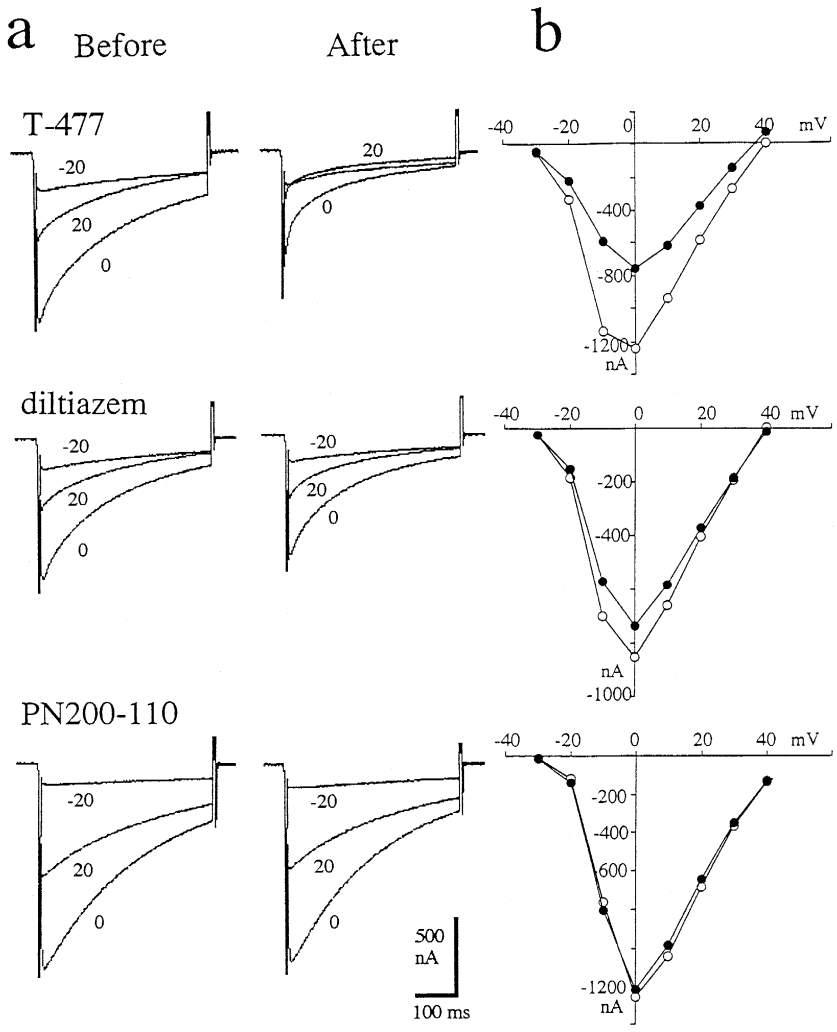


Fig. 4. Effects of Ca^{2+} channel antagonists on cloned brain BI Ca^{2+} channel currents in *Xenopus* oocytes. (a) Original current traces before (left) and after (right) superfusion with 30 μM of T-477 (top), diltiazem (middle), and PN200-110 (bottom). Currents were elicited by 400 ms depolarizing pulses of -20, 0 and 20 mV from a holding potential of -80 mV at an interval of 15 s. (b) The peak current amplitude is plotted as a function of depolarizing step potentials in the absence (○) and the presence (●) of 30 μM of T-477 (top), diltiazem (middle), PN200-110 (bottom).

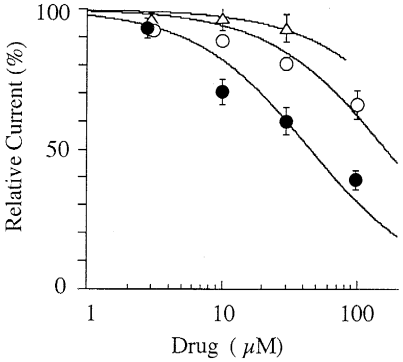


Fig. 5. Dose–response relationship for inhibition of the BI current by T-477 (●), diltiazem (○) and PN200-110 (△). The amplitude of peak current elicited by 400 ms depolarizing pulses of 0 mV from a holding potential of −80 mV at an interval of 15 s was normalized to the control amplitude in the absence of drug. Each point represents the mean ± S.E.M. obtained from 5 experiments.

Measurements were made using a standard two-pulse protocol: A 5 s conditioning pulse to various potential levels was followed by a 300 ms test pulse to 0 mV. As expected from the dose–response curve of Fig. 5, the peak BI current decreased by about 35% at large negative conditioning potentials (−80 mV). When the peak current in the presence of T-477 was normalized to that of control, a slight shift of the curve in the hyperpolarizing direction became apparent. The midpotentials were -42.39 ± 0.47 and -46.32 ± 0.66 mV ($n = 5$, $P < 0.01$) and the slope factors were 6.37 ± 0.23 and 6.94 ± 0.19 ($n = 5$, not significant) before and after application of the drug, respectively. The result excludes the possibility that T-477 strongly affects steady-state inactivation properties.

Next, we examined the influences of repetitive stimulation of depolarizing pulses on the time course and potency of the inhibitory effect of T-477 on BI current. A train of depolarizing pulses to 0 mV from a holding potential of −80 mV was given to the oocytes at a frequency of 0.5 and 2 Hz. Under control conditions with a pulse duration of 100 ms and a frequency of 0.5 Hz, the peak amplitude of BI current at the 10th pulse was $86.7 \pm 4.8\%$ ($n = 3$) of that at the 1st pulse (Fig. 7). In the presence of 30 μM T-477, the current amplitude at the 10th pulse remained $74.7 \pm 6.8\%$ ($n = 3$) of that at the 1st pulse and was not significantly different from control. However, when the frequency was increased to 2 Hz, the drug produced a profound decrease, indicating a use-dependent block. The current amplitudes at the 10th pulse were $67.9 \pm 3.1\%$ ($n = 3$) and $43.6 \pm 1.6\%$ ($n = 3$, $P < 0.005$) of those at the 1st pulse in the absence and presence of drug, respectively.

Many compounds that show a use-dependent block have open-channel blocking action. However, considering its weak block at a low frequency (0.5 Hz), it is unlikely that an augmentation in the inhibitory action of T-477 by train pulses is due to an open-channel blocking action. Therefore, to seek a possible mechanism for such dependence on pulse train frequency, the effect of T-477 on the reactivation of BI channel from inactivation state was examined using a double pulse method. The pair of depolarizing pulses from a holding potential of −80 to 0 mV with various intervals were applied to the oocyte as shown in the inset of Fig. 8. The peak amplitude of the BI current elicited by the second pulse was normalized to that by the

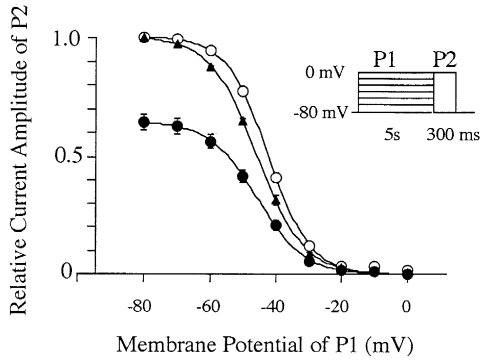


Fig. 6. Effect of T-477 on the voltage-dependent inactivation of the BI current. A standard double-pulse protocol was used (see inset). The oocyte was held at −80 mV and stepped to various levels (P1) for 5 s and then stepped to 0 mV (P2) to activate the BI current. Each double pulse was applied every 30 s. The current amplitude during a test pulse (P2) was normalized to the control amplitude in the absence of conditioning pulse before application of the drug, and is plotted as a function of conditioning potential levels (P1). (○) Before T-477 application; (●) after application of 30 μM T-477; and (▲) the same as (●) but normalized to the control amplitude in the absence of conditioning pulse. Each point represents the mean ± S.E.M. from 5 experiments. Solid lines are drawn according to Boltzman distribution: $I = 1/\{1 + \exp[(V - V_h)/s]\}$, where I is the relative current of P2 and V is the membrane potential of P1. The midpotential, V_h, and the slope factor, s, were −42.4 mV and 6.38, respectively, for the control currents (○) and −45.8 mV and 7.10 in the presence of drug (▲).

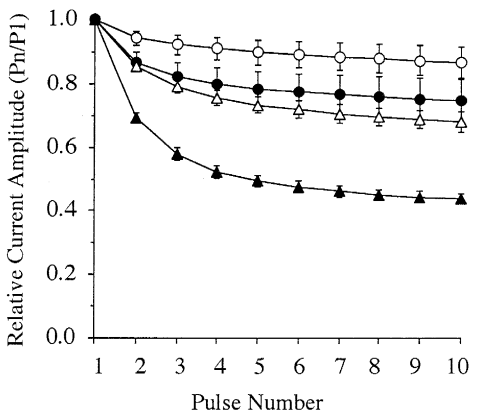


Fig. 7. Use-dependent inhibition of BI current evoked by a 0.5 or 2 Hz train of 100 ms depolarizing pulses of 0 mV from −80 mV in the absence (○, 0.5 Hz; △, 2 Hz) and the presence of 30 μM T-477 (●, 0.5 Hz; ▲, 2 Hz). Peak amplitude of BI current was normalized to that during the first pulse. Each point represents the mean ± S.E.M. from 3 experiments.

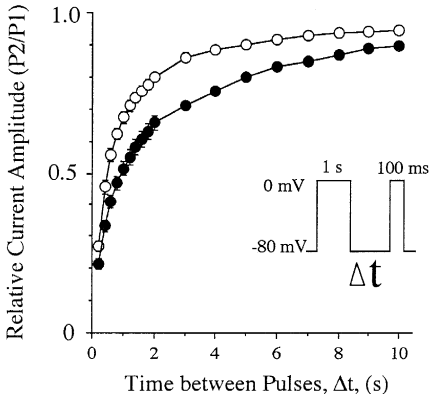


Fig. 8. Recovery of BI channel from the inactivated state in the absence (○) and the presence (●) of 10 μM T-477. Recovery was assessed using the double-pulse protocol shown in the inset. Each double-pulse sequence was given at 30 s intervals. The current amplitude during the test pulse was normalized to that during the conditioning 1 s pulse, and is plotted as a function of the pulse interval. Each point represents the mean ± S.E.M. from 5 (interval of 0–2 s) or 3 (interval of 3–10 s) experiments.

first one, and was plotted against the pulse interval (Fig. 8). BI channels inactivated completely by the first pulse with a duration of 1 s and recovered after the interval. As the pulse interval was prolonged, the relative amplitude of BI current increased; 90% of recovery was observed at an interval of 5 s. In the presence of T-477, the recovery was delayed and the time required for 90% recovery increased to 10 s. Thus, the cumulative inhibitory action of T-477 by repetitive depolarization could be due to a delayed reactivation.

3.4. Effect of T-477 and the classical organic Ca^{2+} channel antagonists on cloned brain Ca^{2+} channel BII and BIII current

The effect of T-477 and diltiazem on other neuronal dihydropyridine-insensitive Ca^{2+} channels such as Ni^{2+}-sensitive R-type and ω-conotoxin-GVIA-sensitive N-type was also tested. Oocytes injected with cRNA specific for BII α_{1E} or BIII α_{1B} subunits exhibited inward Ba^{2+} currents that activated and inactivated rapidly in response to depolarizing voltage clamp pulses of > -30 mV from a holding potential of -80 mV (Fig. 9a). The current amplitude reached a peak at about 10 mV (BII) or 0 mV (BIII) (Fig. 9b).

Extracellular application of T-477 suppressed the BII and BIII currents, without changing the shape of the *I–V* curve (Fig. 9b). The dose–response relationships for T-477, diltiazem and PN200-110 block of BII and BIII currents are illustrated in Fig. 10, where the peak amplitude of the currents in response to depolarizing pulses to 10 mV (BII) or 0 mV (BIII) is plotted as a function of drug concentration. The IC_{50} of T-477 for BII and BIII currents were 74 and 59 μM, respectively, being similar to IC_{50} for BI and L-type α_{1C} currents. However, the inhibition by diltiazem and PN200-110 was weak, in contrast to the strong inhibi-

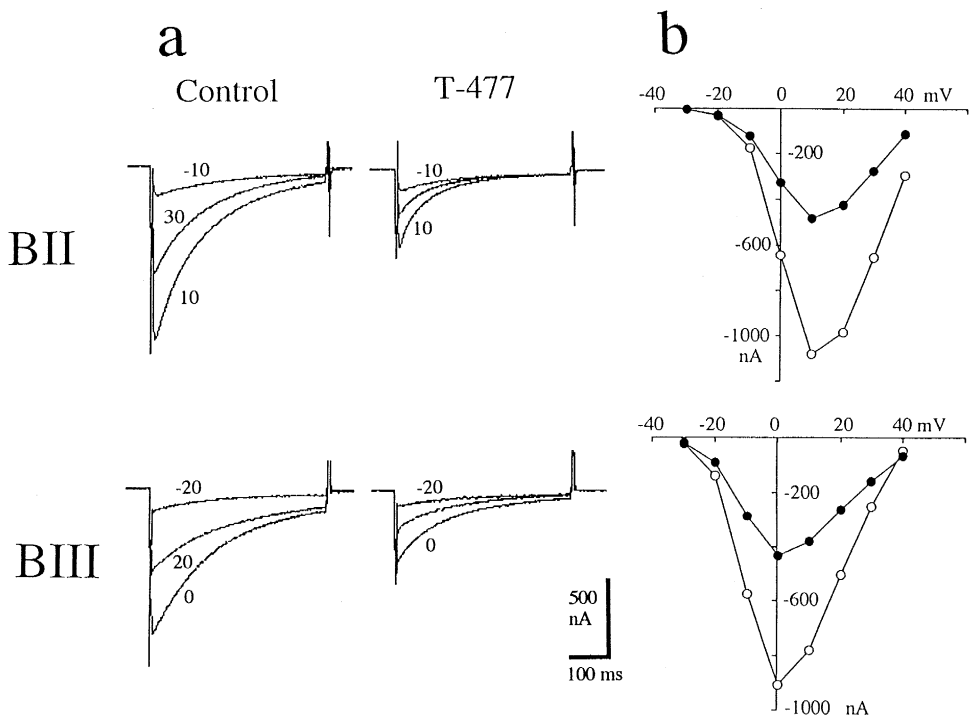


Fig. 9. Effects of T-477 on brain Ca^{2+} channels BII and BIII currents in *Xenopus* oocytes. (a) Original current traces before (left) and after (right) superfusion with 30 μM (BIII) or 100 μM (BII) of T-477. Currents were elicited by 400 ms depolarizing pulses of various levels (−10, 10, 30 mV for BII and −20, 0, 20 mV for BIII) from a holding potential of −80 mV at an interval of 15 s. (b) The peak amplitude of BII (upper) and BIII (lower) current is plotted as a function of depolarizing step potentials in the absence (○) and the presence (●) of 30 μM (BIII) or 100 μM (BII) of T-477.

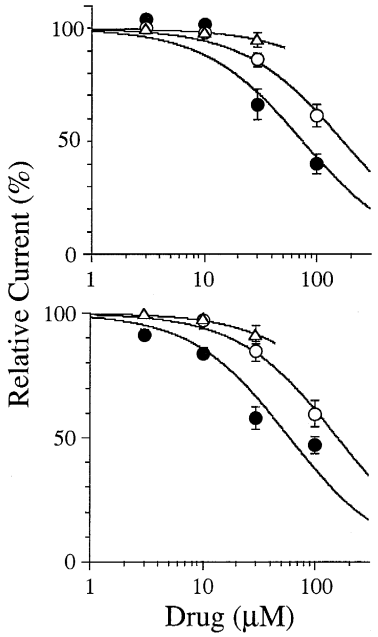


Fig. 10. Dose–response relationship for inhibition of the BII (upper) and BIII (lower) current by T-477 (●), diltiazem (○) and PN200-110 (△). The amplitude of peak current elicited by 400 ms depolarizing pulses of 10 mV (BII) or 0 mV (BIII) from a holding potential of −80 mV at an interval of 15 s was normalized to the control amplitude in the absence of drug. Each point represents the mean ± S.E.M. obtained from 5 experiments.

tion of the L-type α_{1C} channel. The IC_{50} of diltiazem for both currents were 167 and 157 μM, respectively. PN200-110 had no effect at a concentration of 30 μM.

4. Discussion

Our results indicate that Ca^{2+} channel antagonist T-477 inhibits various types of Ca^{2+} channels heterologously expressed in *Xenopus* oocytes. The potency of a blockade by T-477 is not significantly different among the L-type α_{1C}, N-type BIII (α_{1B}), P or Q-type BI (α_{1A}), and R-type BII (α_{1E}) channels, suggesting that the drug is able to suppress fully Ca^{2+} influx generated by VDCCs in neurons.

Some Ca^{2+} channel antagonists such as nimodipine (Steen et al., 1983) and nicardipine (Alps et al., 1987) have been proven to be effective in treatment of stroke. However, it seems unlikely that their effects are due to direct inhibition of neuronal Ca^{2+} influx, because they improve the cerebral circulation by strong inhibition of Ca^{2+} influx in vascular smooth muscle cells. In fact, dihydropyridines and diltiazem have been reported to inhibit VDCC of cardiac and smooth muscle cells, while having little effect on neuronal VDCCs (Hille, 1992). Similarly, in our experiments, diltiazem and the dihydropyridine PN-200-110 also selectively block the L-type α_{1C} channel: IC_{50} values for the blockade of L-channel by the two Ca^{2+} channel antagonists were at least an order of magnitude lower than those for the blockade of the BI channel. As a neuroprotective agent, the inhibitory spectrum of T-477 is unique. The difference in blocking selectivity between chemically analogous Ca^{2+} channel antagonists, diltiazem and T-477, together with their distinctive biological activity, has important implications as to the mechanisms underlying the neuroprotective effects of Ca^{2+} channel antagonists in brain ischemia. Since experimental evidence indicates that T-477 is able to block neuronal non-L-type channels, it is estimated that the block of Ca^{2+} influx through these channels protects neuronal cells from ischemic cell death. However, T-477's ability to block a wide variety of VDCC may indicate that its efficiency in neuroprotection derives from its action on vascular smooth muscle in the brain tissue. This possibility is excluded by the fact that strong vasoactive agents, including diltiazem, which have much higher affinity to smooth muscle L-type α_{1C} channels, show marginal neuroprotective ability. The results overall imply that the high efficiency of Ca^{2+} channel antagonists as neuroprotective agents can be mainly yielded through their direct inhibition of Ca^{2+} influx generated by neuronal VDCCs but not only through inhibition of Ca^{2+} influx in vascular smooth muscle cells. High permeability through the blood–brain barrier, that induces the concentration increase of non-metabolized T-477 up to approximately 50 μM 30 min after i.v. administration (10 mg/kg) in the rat brain (Kudo, unpublished data), and subsequent blockade of neuronal VDCC by T-477, may allow high pharmacotherapeutic efficiency at low doses avoiding serious side effects on cardiovascular systems such as blocking action on the heart seen in conventional Ca^{2+} channel antagonists.

It is not yet determined which type(s) of neuronal VDCC is involved in development of neuronal death during ischemia. Five classes of VDCC α_1 subunits have been cloned from mammalian brain. Two of which are the α_{1C} (so-called 'cardiac type') and α_{1D} subunits that produce dihydropyridine-sensitive L-type VDCC currents. The other three, the BI, BIII and BII α_1 (α_{1A}, α_{1B} and α_{1E}) subunits, correspond to dihydropyridine-insensitive P/Q-, N- and R-type, respectively, that have been defined electrophysiologically and pharmacologically in intact neurons (reviewed by Varadi et al., 1995). Considering the activities of neuronal VDCCs in normal neuronal processes (Tsien and Tsien, 1990; Fujita et al., 1993), it is highly possible that VDCCs play important roles also in pathological pathways. Since the neuroprotective ability of dihydropyridines and diltiazem that specifically display strong inhibitory action on L-type channels is marginal compared to that of T-477 which blocks the P/Q-type BI, N-type BIII and R-type BII channels, the neuroprotective effect of T-477 may not be due to block of the L-type α_{1C} and α_{1D} channels in the neuronal cells. Of the other three channels, blockade of the BI α_{1A} channels, that induce glutamate release as P/Q-type channels (Turner et al., 1992; Gaur et

al., 1994), by T-477 should be noted because it is abundant in cerebellar Purkinje cells (Mori et al., 1991) which are selectively vulnerable to ischemia as well as hippocampus (Wieloch, 1985). Thus, it is conceivable that non-L-type neuronal Ca^{2+} channels are mainly responsible for neuronal death in brain ischemia.

We also studied the mechanism of T-477 action on the BI Ca^{2+} channel. The steady-state inactivation curves for BI channels before and after exposure to 30 μM T-477 (Fig. 6) did not exhibit significant differences. This indicates that T-477 does not significantly affect the steady-state inactivation properties of the channel. The influence of repetitive stimulation of depolarizing pulses on the time course and potency of the inhibitory effect of T-477 on BI current was also examined. When the frequency was increased from 0.5 to 2 Hz, the drug produced a profound decrease in current amplitude, typical of a use-dependent block. Although many compounds that show a use-dependent block have open-channel blocking action, it is unlikely that an augmentation in the inhibitory action of T-477 by train pulses is due to an open-channel blocking action, considering its weak block at a low frequency (0.5 Hz). A double pulse method with various intervals (Fig. 8) indicates that twice the length of time is necessary for the 90% recovery of the BI current in the presence of T-477. It is therefore possible that T-477 delays the recovery from the inactivated state of the BI channel by elevating the free energy level of (destabilizing) the transition state between the inactivated and resting state. T-477's ability to be more effective at channels that are frequently excited may be helpful in producing its neuroprotective effect during excessive activity in stroke or epileptic seizure.

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