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Effects of a novel, potent benzothiazepine Ca^{2+} channel antagonist, DTZ323, on guinea-pig ventricular myocytes

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Abstract

The effects of a 1,5-benzothiazepine derivative, (+)-*cis*-3-(acetyloxy)-5-[2-[[2-(3,4-dimethoxyphenyl)ethyl]-methylamino]ethyl]-2,3-dihydro-2-(4-methyoxyphenyl)-1,5-benzothiazepine-4(5*H*)-one (DTZ323), on membrane currents were investigated in guinea-pig ventricular myocytes using the whole-cell patch-clamp technique. DTZ323 suppressed the L-type Ca^{2+} channel currents ($I_{Ca(L)}$) more selectively than the T-type Ca^{2+} channel and the Na^{+} channel currents. DTZ323 inhibited $I_{Ca(L)}$ in a use- and a voltage-dependent manner with 24 times higher potency than that of diltiazem. Rate of recovery of $I_{Ca(L)}$ from the conditioned block by DTZ323 was faster compared with diltiazem and verapamil, and was steeply dependent on the holding potential at resting membrane potential range in ventricular myocytes (−90 to −60 mV). Our results suggest that DTZ323 is a selective Ca^{2+} channel antagonist, the most potent among the 1,5-benzothiazepine Ca^{2+} channel antagonists, and that the voltage- and use-dependent effect of DTZ323 on $I_{Ca(L)}$ is due to the steep voltage dependence of the rate of dissociation from the cardiac L-type Ca^{2+} channels.

Keywords: Benzothiazepine; Diltiazem; Verapamil; Ca^{2+} channel antagonist; Ventricular myocyte; Ca^{2+} channel, L-type

1. Introduction

Ca^{2+} channel antagonists are classified into three major chemical classes, the 1,4-dihydropyridines, phenylalkylamines and 1,5-benzothiazepines. In addition, several unrelated new chemicals have been identified as Ca^{2+} channel antagonists. It is widely accepted that these chemical classes of Ca^{2+} channel antagonists have distinct but allosterically interacting binding sites within the α_1 subunit of the L-type Ca^{2+} channel (McDonald et al., 1994). Each class of Ca^{2+} channel antagonists suppresses the L-type Ca^{2+} channel current ($I_{Ca(L)}$) in a distinctive manner, and has unique tissue selectivity (Taira, 1987; Triggle, 1991; Spedding and Paoletti, 1992).

A novel 1,5-benzothiazepine derivative, (+)-*cis*-3-(acetyloxy)-5-[2-[[2-(3,4-dimethoxyphenyl)ethyl]-methylamino]-ethyl]-2,3-dihydro-2-(4-methyoxyphenyl)-1,5-benzothiazepine-4(5*H*)-one (DTZ323, see Fig. 1) with a pK_a value of 6.1, has been recently introduced (Hagiwara et al., 1997). In rabbit crude T-tubule membranes, DTZ323 has been shown to produce complete inhibition of [^{3}H]diltiazem binding with a Hill coefficient close to unity, indicating competitive inhibition. DTZ323 (K_i 6.6×10^{-9} M) was 48 times more potent than diltiazem (K_i 3.1×10^{-7} M) and nine times more potent than clentiazem (K_i 6.1×10^{-8} M) (Hagiwara et al., 1997). Thus, DTZ323 has the highest affinity for 1,4-dihydropyridine receptors of skeletal muscle membrane of all the 1,5-benzothiazepine derivatives studied to date.

The aim of this study was to characterize pharmacological effects of DTZ323 on cardiac ion channels. In the present study, we used the *d-cis* isomer of DTZ323, because the effects of diltiazem on the radioligand binding to Ca^{2+} channels were highly stereospecific to the *d-cis* isomer (Ikeda et al., 1991).

Phenylalkylamine and 1,5-benzothiazepine compounds have been reported to suppress $I_{Ca(L)}$ in a use-dependent manner (McDonald et al., 1984; Kanaya and Katzung, 1984). Dihydropyridines also show the use-dependent block at high frequencies, depending on the ionization constants of the compounds (Uehara and Hume, 1985; Sanguinetti and Kass, 1984). We have reported that the negative inotropic effect of diltiazem is dramatically augmented by the slight change of the holding potential from −80 mV to

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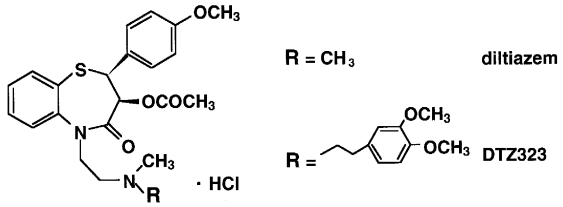

Fig. 1. Structures of 1,5-benzothiazepine derivatives studied.

−60 mV, which is very close to the change of the resting membrane potential under ischemic condition, and that such augmentation of the effect of diltiazem is explained by its voltage dependence of the use-dependent block of $I_{Ca(L)}$ (Okuyama et al., 1994).

Ca^{2+} channel antagonists which act in a use-dependent manner require the activation of Ca^{2+} channels to gain access to their binding sites either in the open state or in the inactivated state following the opening of Ca^{2+} channels. Since Ca^{2+} channel antagonists prolong the inactivated state of the channel (McDonald et al., 1994), high frequency of depolarizing pulses accelerates the shift of Ca^{2+} channel gating to the inactivated state. The use-dependent block develops more rapidly and more strongly at depolarized holding potentials, which is explained by the voltage dependence of dissociation rates of the drugs (McDonald et al., 1994). Recovery from the block of $I_{Ca(L)}$ has been investigated for 1,4-dihydropyridines and phenylalkylamine Ca^{2+} channel antagonists in cardiac tissue, which was fast for nisoldipine ($\tau 1 = 1.5$ s, $\tau 2 = 30$ s; Sanguinetti and Kass, 1984) and was slow for D600 ($\tau = 2.4$ min; McDonald et al., 1984) at the same holding potential (−70 mV). The difference of the rate of recovery between 1,4-dihydropyridine and phenylalkylamine is reflected in the difference of the use-dependent block of $I_{Ca(L)}$ between them, namely, slow recovery enhances the use-dependent block.

In the present study, we examined the pharmacological properties of DTZ323, and investigated the manner of suppression of $I_{Ca(L)}$ by DTZ323 in comparison with diltiazem and verapamil. We found that DTZ323 binds with high affinity to the L-type Ca^{2+} channel and that its affinity is steeply dependent on the membrane potential.

2. Materials and methods

2.1. Preparation of single cells

Ventricular myocytes were isolated enzymatically from the hearts of male Hartley guinea pigs (weight 200–500 g) according to the method described by Cavalié et al. (1983). The animals were anaesthetized with sodium pentobarbital (40 mg/kg) and the ascending aorta was cannulated in situ under artificial respiration. The heart was excised and perfused at 37°C via a Langendorff apparatus, first with a nominally Ca^{2+}-free solution for 10 min, followed by collagenase solution for 7–15 min. Subsequently, the enzyme solution was washed out with a high-K^+, Ca^{2+}-free solution (KB (Kraftbrühe) solution, Isenberg and Klöckner, 1982). The KB solution contained (mM): potassium glutamate 70, KCl 25, oxalic acid 10, KH_2PO_4 10, taurine 10, glucose 11, HEPES 10, EGTA 0.5 (pH 7.4, adjusted with KOH). The ventricular myocytes were dissociated by gentle stirring of the tissue fragments at 37°C. The dissociated cells were stored in KB solution at 4°C, and were used for experiments within 12 h. The normal Tyrode solution used in this procedure had the following composition (mM): NaCl 135, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 11, HEPES 5 (pH 7.4). Ca^{2+} was omitted in Ca^{2+}-free Tyrode solution. The collagenase solution was prepared by adding 70–80 units/ml collagenase (Collagenase S-I) and 14.4 μM $CaCl_2$ to Ca^{2+}-free Tyrode solution.

2.2. Electrophysiology

Currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). The cells were placed in a chamber (volume 0.3 ml) attached to the stage of an inverted microscope (IMT-2, Olympus). The chamber was continuously perfused with modified Tyrode solution with the following composition (mM): NaCl 120, KCl 4, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, HEPES 5 (pH 7.4 adjusted with NaOH) (Shuba et al., 1990). The tip resistance of the heat-polished microelectrodes (borosilicate glass) was 1–5 MΩ when filled with the internal solution composed of (in mM): CsCl 80, CsOH 40, MgATP 5, EGTA 10, HEPES 10 (pH 7.4, adjusted with HCl). After gigaseal formation and rupture of the membrane patch, the external solution was exchanged for Na^+,K^+-free solution with the following composition (mM): tetraethylammonium chloride (TEACl) 120, CsOH 4, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10, HEPES 5 (pH 7.4 adjusted with HCl). Na^+ currents were measured with the external solution containing (in mM): tetraethylammonium chloride 100, NaCl 20, CsOH 4, $CaCl_2$ 2, $MgCl_2$ 2, $CdCl_2$ 0.1, glucose 10, HEPES 5 (pH 7.4 adjusted with HCl). Series resistance was approximately within three times tip resistance of the patch electrodes, and was electrically compensated. Membrane capacitances of the cells used in this study ranged from 150 to 200 pF. $I_{Ca(L)}$ and the T-type Ca^{2+} channel currents ($I_{Ca(T)}$) were measured at room temperature. The Na^+ channel currents (I_{Na}) were measured at 16°C.

Transmembrane currents were obtained using an Axopatch 1C amplifier (Axon Instruments, CA, USA) with a 100-MΩ headstage, low-pass filtered at 1 or 2 kHz, digitized at 10 kHz and sampled on-line to a computer hard disk. The pCLAMP software Ver. 5.5 (Axon Instruments, CA, USA) was used both to generate the voltage-clamp protocols and to acquire data.

2.3. Data analysis

All data were analyzed using pCLAMP software. Peak detection was performed by Clampan programs, and the rate of inactivation of $I_{Ca(L)}$ was calculated by Clampfit programs. When $I_{Ca(L)}$ was measured, leak subtraction was performed either by subtracting the current measured in the presence of 0.1 mM $CdCl_2$ from the raw currents, or by using the P/-4 protocol. When $I_{Ca(T)}$ was measured, leak currents were measured in the presence of 0.1 mM $NiCl_2$ and subtracted from the recorded currents. I_{Na} was measured with leak subtraction using the P/-4 protocol.

Each steady-state inactivation curve was fitted to Boltzmann distribution equations using the Sigma plot program (Jandel Scientific, CA, USA). Curve fitting for the dose-response curve was performed and IC_{50} values were calculated using GraphPad Prism (GraphPad Software, CA, USA). Curve fitting (Quasi-Newton method) for time course of the development of the use-dependent inhibition of $I_{Ca(L)}$ and the rate of recovery of $I_{Ca(L)}$ from the block was performed using the Mac Curve Fit program (custom made by K. Raner).

Statistical significance was assessed with Student's *t*-test for simple comparisons or with Bonferroni's multiple *t*-test for multiple comparisons; differences at $P < 0.05$ were considered to be significant.

2.4. Materials

Diltiazem HCl and DTZ323 were kindly supplied by Tanabe Seiyaku Co. (Saitama, Japan). The structure of DTZ323 is shown in Fig. 1. Verapamil HCl was purchased from Nakalai Tesque (Tokyo, Japan). Collagenase S-I was purchased from Nitta Gelatin (Osaka, Japan).

3. Results

3.1. Effects of DTZ323 on various ionic channels

Effects of DTZ323 on $I_{Ca(T)}$, $I_{Ca(L)}$ and I_{Na} were compared. $I_{Ca(L)}$ and $I_{Ca(T)}$ were isolated by exploiting the large difference in their steady-state inactivation (Balke et al., 1992). I_{Na} was abolished by replacing extracellular Na^+ with TEA^+. Current-voltage (I-V) relationships were investigated using holding potentials of −90 mV and −50 mV (Fig. 2). Depolarizing test pulses for 200 ms were applied in 10 mV steps up to +40 mV at a low frequency of 0.067 Hz. As shown in Fig. 2, the I-V relationships from a holding potential of −50 mV (closed circles in Fig. 2) produced U-shaped curves, indicating a single component of $I_{Ca(L)}$, while the I-V relationships from a holding potential of −90 mV (open circles in Fig. 2) gave two components. These two components represent both $I_{Ca(T)}$ and $I_{Ca(L)}$. The difference between the currents activated at −90 mV and −50 mV (open circles and closed circles, respectively, in Fig. 2) therefore represents $I_{Ca(T)}$ component (open squares in Fig. 2). Based on the results shown in Fig. 2, we elicited $I_{Ca(T)}$ by giving depolarizing pulses (200 ms) to −30 mV from a holding potential of −90 mV, and $I_{Ca(L)}$ with depolarizing pulses to +10 mV from a holding potential of −50 mV in the following experiments.

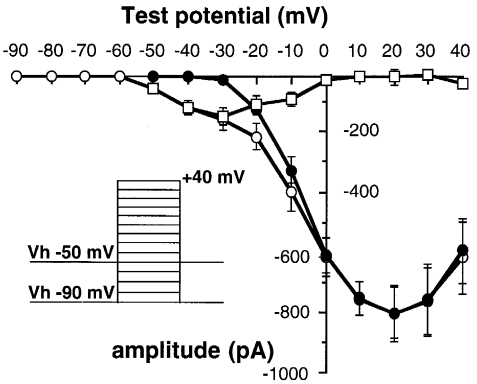

Fig. 2. $I-V$ relationships for the two I_{Ca} components. Currents were elicited from holding potentials of −90 mV (○), at which both T- and L-type Ca^{2+} channels are available to open by depolarization, and from −50 mV (●) at which only L-type Ca^{2+} channels are fully available. Voltage protocols are shown in the inset. $I_{Ca(T)}$ (□) was obtained by subtraction of (●) from (○). Each point represents mean ± S.E.M. of 5 experiments.

Fig. 3a and 3b show effects of DTZ323 (10^{-6} M) on $I_{Ca(L)}$ and $I_{Ca(T)}$, respectively. Both $I_{Ca(L)}$ and $I_{Ca(T)}$ were recorded at a frequency of 0.2 Hz. After 4-min exposure to DTZ323, the peak amplitude of $I_{Ca(L)}$ decreased to less than 20% of the control value (Fig. 3a), while the peak amplitude of $I_{Ca(T)}$ decreased to 92% of the control value (Fig. 3b). DTZ323 (10^{-5} M) suppressed the peak amplitude of $I_{Ca(T)}$ to 51% (data not shown).

Fig. 3c shows the effect of DTZ323 on I_{Na} measured by giving depolarizing pulses for 15 ms to −20 mV from a holding potential of −90 mV at 2 Hz and 3 Hz for 15 ~ 20 min. In the presence of DTZ323 (10^{-5} M), an extremely high concentration (10^{-5} M) which is 10 times higher than the concentration to cause complete block of $I_{Ca(L)}$, the amplitude of I_{Na} decreased to 89.3 ± 3.3% ($n = 12$) of control value when pulsed at 2 Hz and 76.2 ± 6.4% ($n = 7$) at 3 Hz, respectively. In the absence of DTZ323, the amplitude of I_{Na} was not changed at 2 Hz nor at 3 Hz. These results show that high concentration of DTZ323 blocks I_{Na} when I_{Na} is activated at high frequency (> 3 Hz).

In the following series of experiments, we examined effects of DTZ323 on $I_{Ca(L)}$ (Fig. 4). The concentration-response curve for DTZ323 was obtained by measuring the peak amplitude of $I_{Ca(L)}$ after $I_{Ca(L)}$ activated at 0.2 Hz had reached its steady-state level in the presence of the given concentration of DTZ323 (within 10 min). $I_{Ca(L)}$ were elicited by depolarizing pulses for 200 ms to +10 mV from a holding potential of −50 mV. The resulting IC_{50}

value was $(2.57 \pm 0.03) \times 10^{-8}$ M ($n = 5$, $n_H = 0.56$). Diltiazem and verapamil also suppressed $I_{Ca(L)}$ with IC_{50} values of $(6.31 \pm 0.05) \times 10^{-7}$ M ($n = 5$, $n_H = 0.77$) and $(1.64 \pm 0.01) \times 10^{-7}$ M ($n = 5$, $n_H = 0.61$), respectively, under the same condition.

To determine the effects of DTZ323 on the I-V relationships of $I_{Ca(L)}$, depolarizing pulses for 200 ms to potentials between -50 and $+40$ mV were applied in 10 mV steps from a holding potential of -50 mV at 0.033 Hz (Fig. 4b). Data were obtained in the absence (open circles) and presence of DTZ323 (10^{-7} M, closed circles) after the use-dependent blocking action, which was developed by giving test pulses from -50 to 0 mV at 0.067 Hz, had reached steady state. The step test pulses were given at 0.033 Hz, which, we had confirmed, allowed neither further accumulation of the use-dependent block by DTZ323 nor recovery from the blockade at this holding potential. DTZ323 decreased the amplitude of $I_{Ca(L)}$ without changing the voltage-dependence of the I-V relationship. The same extent of blockade of $I_{Ca(L)}$ by 10^{-7} M DTZ323 in Fig. 4a and Fig. 4b infers that the recovery from the blockade by DTZ323 is apparently slow at V_h -50 mV.

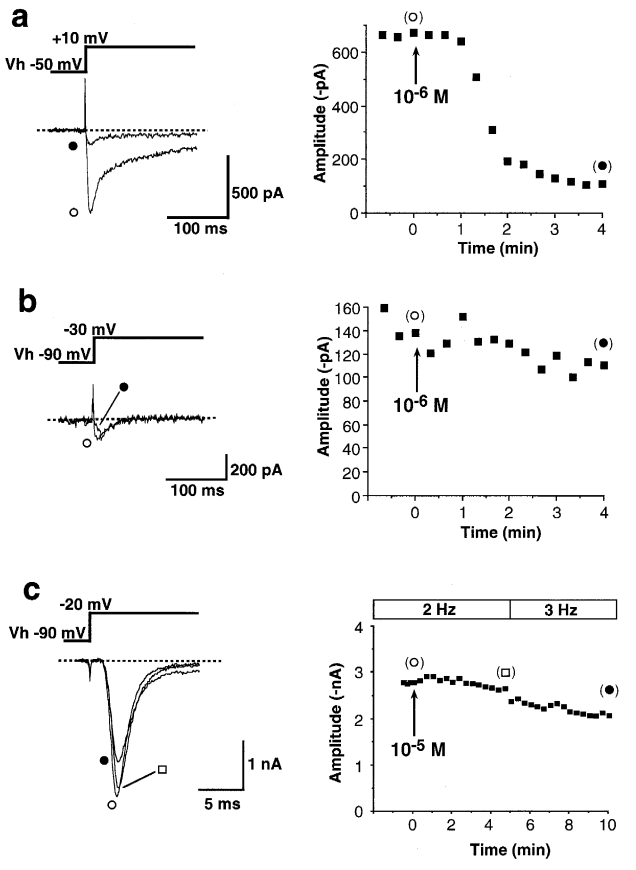

Fig. 3. Effects of DTZ323 on Ca^{2+} channel currents ($I_{Ca(L)}$, $I_{Ca(T)}$) and the Na^+ channel current (I_{Na}). The current traces shown were plotted after subtraction of the leak currents. Records of (a) $I_{Ca(L)}$ and (b) $I_{Ca(T)}$ were obtained before (○) and 4 min after (●) exposure (arrows) of the myocyte to DTZ323 at 10^{-6} M. (c) Records of I_{Na} obtained before (○) and 5 min after exposure (arrows) to DTZ323 at 10^{-5} M pulsed at 2 Hz (□), and 5 min after changing the frequency to 3 Hz (●).

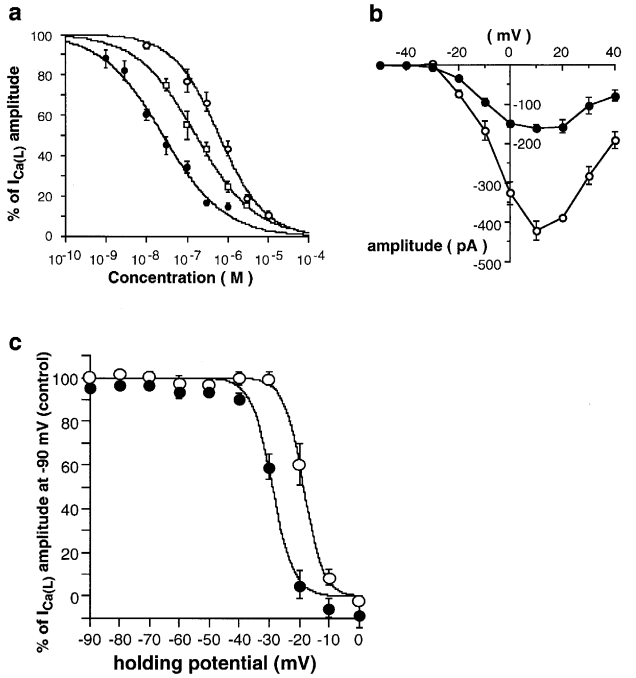

Fig. 4. Effects of DTZ323 on the amplitude of $I_{Ca(L)}$. (a) Dose-dependent inhibition of $I_{Ca(L)}$ by the extracellular application of DTZ323 (●), diltiazem (○) and verapamil (□). The amplitude of $I_{Ca(L)}$ evoked by command pulses in the absence of drugs is represented as 100%. Each point represents mean ± S.E.M. of 5 experiments. (b) Effect of DTZ323 10^{-7} M on I-V relationships. Two records of the inward currents before (○) and after (●) application of the drug were superimposed. Each plot represents mean ± S.E.M. of 5 experiments. (c) Effect of DTZ323 10^{-6} M on the voltage dependence of Ca^{2+} current availability. Steady-state inactivation curves were obtained before (○) and after exposure to DTZ323 (●). Values represent mean ± S.E.M. of 5 experiments.

The interaction between Ca^{2+} channel antagonists and L-type Ca^+ channels is known to be modulated by the state of the channel or by the transmembrane potential, thus showing voltage dependence, use dependence, or both. The effects of DTZ323 (10^{-6} M) on the voltage dependence of the availability of L-type Ca^{2+} channels are shown in Fig. 4c. The availability of L-type Ca^{2+} channels was determined by a double-pulse protocol at a frequency of 0.033 Hz. Membrane potential was stepped to various potentials for 20 s from a holding potential at -90 mV, then followed by a test pulse to $+10$ mV for 200 ms. The peak $I_{Ca(L)}$ amplitudes were normalized relative to that of $I_{Ca(L)}$ recorded in the absence of DTZ323 at -90 mV. Application of DTZ323 for 3 min produced no tonic block at -90 mV, suggesting that DTZ323 hardly binds to the resting state of the channel. Curves in Fig. 4c were fitted to the Boltzmann's distribution: $(I/I_{max} = \{1 + \exp[(V_{0.5} - V)/k]\}^{-1})$, where $V_{0.5}$ is the mid potential and k is the slope of the curve. Mean control values for $V_{0.5}$ and k in five cells were -18.2 ± 1.4 mV and 2.3 ± 0.4 (mean ± S.E.M.), respectively. Application of DTZ323 shifted the $V_{0.5}$ value to -27.9 ± 1.1 mV (Student's t-test, $P < 0.01$) with the same slope factor ($k = 2.7 \pm 0.5$) as the control.

DTZ323 at 10^{-6} M significantly shifted $V_{0.5}$ value towards a negative potential by 9.8 mV.

3.2. Use-dependent block of $I_{Ca(L)}$ by DTZ323

In this series of experiments, myocytes were exposed to DTZ323 for 3 min without test pulses, and then $I_{Ca(L)}$ were elicited at 0.2 Hz in the presence of DTZ323. DTZ323 suppressed $I_{Ca(L)}$ in a use-dependent manner when $I_{Ca(L)}$ were evoked from a holding potential at −50 mV, but not at −90 mV (Fig. 5a). The degree of the use-dependent block, therefore, depended on the holding potential, as is the case with other Ca^{2+} channel antagonists. The peak amplitudes of the first $I_{Ca(L)}$ evoked in the presence of DTZ323 were the same as those of control at both −50 mV and −90 mV, suggesting that no tonic block took place at either potential. The lack of tonic block indicates that the rested state has no significant affinity for the drug.

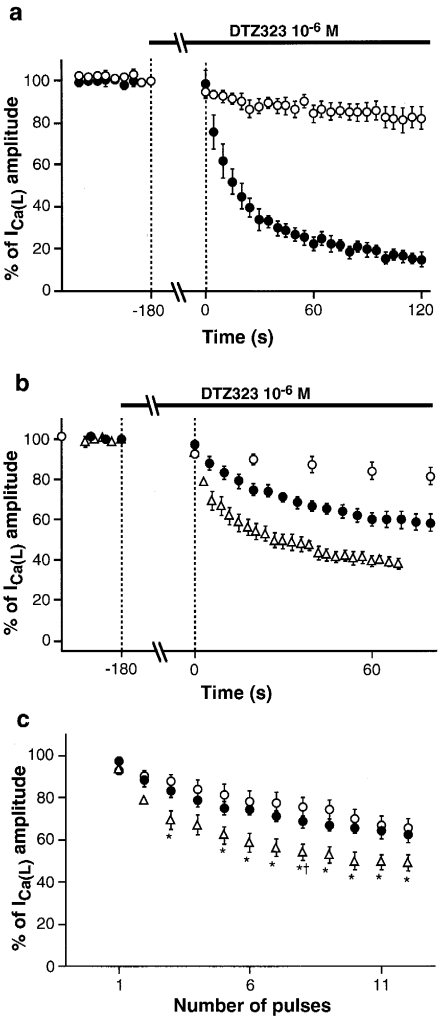

Fig. 5. Use-dependent block induced by DTZ323. Records were obtained 3 min after drug application. Data were normalized to the amplitude of $I_{Ca(L)}$ just before the drug application. (a) Voltage dependence of the use-dependent block induced by DTZ323 10^{-6} M. Currents were elicited by voltage pulses (0.2 Hz) to +10 mV from holding potentials of −50 mV (●) and −90 mV (○). (b) Frequency-dependent block induced by DTZ323 10^{-6} M. Currents were elicited by voltage pulses (0.05 Hz ○, 0.2 Hz ●, 0.33 Hz △) to +10 mV from a holding potential of −70 mV. (c) The results shown in (b) were re-plotted as a function of the number of test pulses. * $P < 0.05$ versus 0.05 Hz; † $P < 0.05$ versus 0.2 Hz (Bonferroni's multiple *t*-test). Values represent mean ± S.E.M.; $n = 5$.

To examine whether the blocking effect of DTZ323 develops depending on the opening of the channel, we investigated the frequency dependence (0.33, 0.2 and 0.05 Hz) of the use-dependent block by DTZ323 (10^{-6} M) at a holding potential of −70 mV, where the speed of unblock is appropriate to examine this effect (Fig. 5b and c). The depolarizing pulses given at these frequency did not change the amplitude of $I_{Ca(L)}$ in the absence of drug. We did not perform the experiment at a frequency higher than 0.4 Hz, because the amplitude of $I_{Ca(L)}$ was depressed to less than 80% of control (data not shown). The rate of the use-dependent decay of $I_{Ca(L)}$ in the presence of DTZ323 varied depending on the frequency of the train pulses (Fig. 5b). The time constants of the decay were 28.77 ± 3.22 s at 0.33 Hz, 103.0 ± 12.9 s at 0.2 Hz and 392.7 ± 66.9 s at 0.05 Hz (Bonferroni's multiple *t*-test, 0.33 Hz vs. 0.05 Hz, $P < 0.01$; 0.2 Hz vs. 0.05 Hz, $P < 0.01$). When the data in Fig. 5b were plotted against the number of pulses (Fig. 5c), the blockade of $I_{Ca(L)}$ by DTZ323 developed significantly faster at 0.33 Hz than at 0.2 Hz or 0.05 Hz. These results suggest that the blocking action is not dependent on the opening of Ca^{2+} channels, but is dependent on the frequency of test pulses, and that DTZ323 stabilizes Ca^{2+} channels in the inactivated state. If DTZ323 binds exclusively to the open channels, the acceleration of the rate of inactivation may be observed. Thus, we analyzed the rate of inactivation of $I_{Ca(L)}$ recorded before and after application of DTZ323. DTZ323 at 10^{-6} M did not alter the rate of inactivation of $I_{Ca(L)}$ significantly (control; $\tau 1 = 9.3 \pm 1.7$ ms, $\tau 2 = 107.4 \pm 6.2$ ms vs. DTZ323; $\tau 1 = 7.4 \pm 0.8$ ms, $\tau 2 = 134.8 \pm 16.8$ ms, $n = 10$).

3.3. Recovery from block of $I_{Ca(L)}$ by DTZ323, diltiazem and verapamil

In order to clarify the voltage dependence of the use-dependent effect of DTZ323, we examined the recovery of $I_{Ca(L)}$ from the block by DTZ323 in comparison with diltiazem and verapamil.

Fig. 6 shows the blockade of $I_{Ca(L)}$ produced by depolarizing pulses and the recovery of $I_{Ca(L)}$ by giving hyperpolarizing pulses (unblock pulses) in the presence of DTZ323 10^{-6} M. The experimental protocol is shown in Fig. 6a. After establishing the blockade of $I_{Ca(L)}$ by drugs at a holding potential of −50 mV (blocking period), myocytes were held at −90 mV for 90 s to allow unblock (unblocking period). The holding potential was changed to −50 mV, and 50 ms later, the block was re-imposed by giving 25 depolarizing pulses to +10 mV at 0.2 Hz (reference period). The cell was exposed to the unblocking period at each voltage (unblocking test voltage, $T_{unblock}$)

for various durations (t), then depolarizing test pulses were applied at a holding potential of −50 mV (0.2 Hz) to quantify the recovery (test period). At the end of the experiment, an unblocking period (90 s, −90 mV) and a depolarizing pulse were applied again to check the degree of 'rundown' of $I_{Ca(L)}$. The time course of a typical experiment is summarized in Fig. 6b, and the current traces recorded at the times indicated by asterisks are shown in Fig. 6c. In this typical experiment, unblock was performed at −70 mV for 6 s ($T_{unblock} = -70$ mV, $t = 6$ s). The recovery of $I_{Ca(L)}$ after $T_{unblock}$ at −90 mV for 6 s is presented in Fig. 6d. The degree of recovery made by $T_{unblock}$ at −70 mV (Fig. 6c) was less than that caused by $T_{unblock}$ at more negative voltage, −90 mV (Fig. 6d).

Rates of recovery of $I_{Ca(L)}$ from the conditioned block by DTZ323 at different $T_{unblock}$ (−90 mV, −70 mV, −60 mV and −50 mV) are summarized in comparison with those produced by diltiazem and verapamil (Fig. 7). The concentration of each drug was chosen based on the concentration-response curve summarized in Fig. 4a (DTZ323 10^{-6} M, diltiazem 2×10^{-5} M and verapamil 5×10^{-6} M, respectively). The proportion of $I_{Ca(L)}$ which recovered during the unblock test period (at $T_{unblock}$ for t) was calculated with reference to $I_{Ca(L)}$ recovered during the first unblocking period (at −90 mV for 90 s). Thus the fraction of block remaining after the unblock test period was calculated with the following equation: $1 - [I_{Ca.test} - I_{Ca.before\,UB\text{-}test}]/[I_{Ca.ref.first} - I_{Ca.before\,UB}]$, where $I_{Ca.test}$ represents the amplitude of the first $I_{Ca(L)}$ measured at the beginning of the test period, $I_{Ca.before\,UB\text{-}test}$ represents the amplitude of $I_{Ca(L)}$ measured at the end of the reference period, $I_{Ca.ref.first}$ represents the amplitudes of the first $I_{Ca(L)}$ measured of the reference period, and $I_{Ca.before\,UB}$ represents the amplitude of $I_{Ca(L)}$ measured at the end of the blocking period.

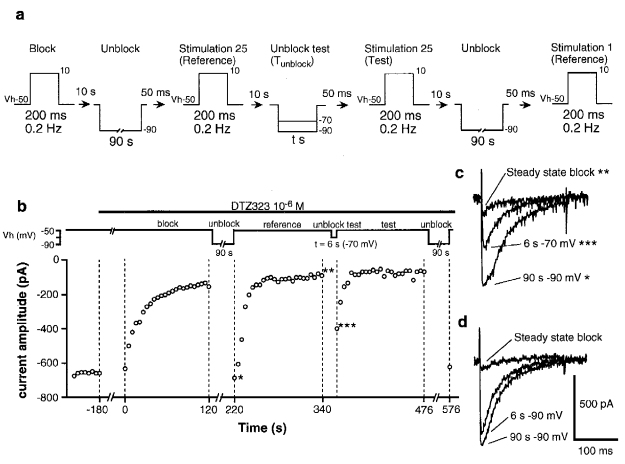

Fig. 6. Voltage dependence of recovery from the conditioned block. (a) The voltage-clamp protocol. (b) Time course of a typical experiment ($t = 6$ s, −70 mV). (c) Superimposed current traces recorded at the respective point indicated by asterisks in (b): $I_{Ca(L)}$ recorded after 90 s of unblock at −90 mV (∗), after blockade reached steady state (∗∗), and after 6 s of unblock at −70 mV (∗∗∗). (d) Superimposed traces of $I_{Ca(L)}$ after 90 s of unblock at −90 mV, after blockade had reached steady state, and after 6 s of unblock at −90 mV.

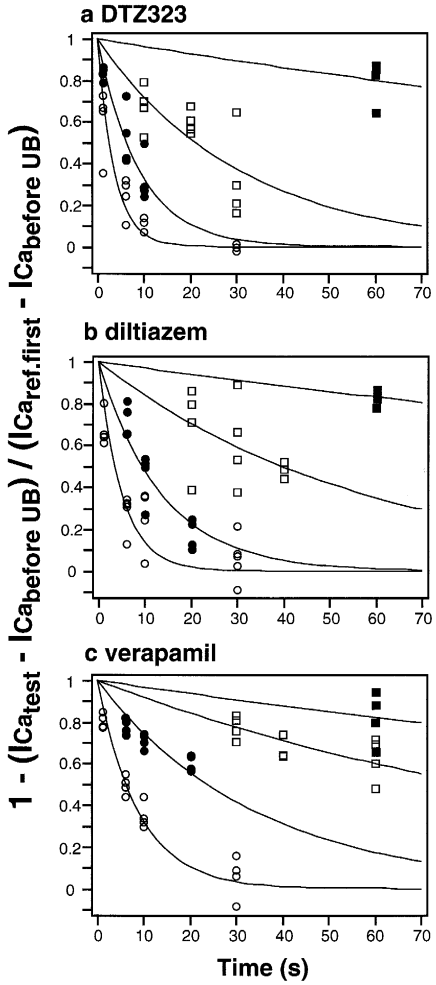

Fig. 7. Time- and voltage-dependent recovery from the block of $I_{Ca(L)}$ by DTZ323 10^{-6} M (a), diltiazem 2×10^{-5} M (b), and verapamil 5×10^{-6} M (c). The procedure is the same as shown in Fig. 6. The fraction of block of $I_{Ca(L)}$ remaining after the unblock test period was calculated as $1 - [I_{Ca.test} - I_{Ca.before\,UB}]/[I_{Ca.ref.first} - I_{Ca.before\,UB}]$ (see Section 3), and plotted as a function of the duration of the unblocking pulse (t). Symbols ($T_{unblock}$): ○, −90 mV; ●, −70 mV; □, −60 mV; ■, −50 mV.

Recovery of $I_{Ca(L)}$ from the blockade by all three drugs was voltage dependent. As is shown in Fig. 7, the recovery from the conditioned block by DTZ323 was fitted to a single exponential. The half-recovery times are summarized in Table 1. The recovery from the block by DTZ323

Table 1
Effects of DTZ323, diltiazem and verapamil on the half-recovery times of unblock

	Unblocking potential		
	−90 mV	−70 mV	−60 mV
DTZ323	2.49 ± 0.35	6.24 ± 0.58	21.1 ± 2.46
Diltiazem	3.51 ± 0.41	9.40 ± 0.79	39.7 ± 5.7
Verapamil	6.14 ± 0.42	23.7 ± 1.6	81.8 ± 6.8

Each value is given in s and represents mean ± S.E.M. ($n = 3 \sim 4$).

was the fastest followed by diltiazem and verapamil at a voltage range of −90 mV to −60 mV. At more positive voltage (−50 mV), the rates of the recovery were too slow to determine reliable half-recovery times. Percentages of recovery after 60 s at −50 mV were 19.9 ± 4.5% with DTZ323, 16.8 ± 1.7% with diltiazem and 17.7 ± 5.6% with verapamil, respectively ($n = 4$, % of control).

4. Discussion

In the present study, we found that DTZ323 is highly selective for L-type Ca^{2+} channels. DTZ323 (10^{-5} M) partially blocked I_{Na} when Na^+ channels were activated at a frequency higher than 3 Hz, indicating rapid dissociation of DTZ323 from Na^+ channels. The compound suppressed $I_{Ca(T)}$ slightly at 10^{-6} M, and blocked it to about 50% of the control value at 10^{-5} M. Thus, the selectivity of DTZ323 for L-type Ca^{2+} channels is ~100 times higher than that for T-type Ca^{2+} channels. These results demonstrate that DTZ323 acts primarily on L-type Ca^{2+} channels.

In [^{3}H]diltiazem binding experiments using rabbit skeletal muscle membranes, DTZ323 was 48 times more potent than diltiazem (Hagiwara et al., 1997). In this electrophysiological study using cardiac myocytes, DTZ323 was 24 times more potent than diltiazem. These potency ratios correlate well, suggesting that DTZ323 inhibits $I_{Ca(L)}$ by binding to the L-type Ca^{2+} channel.

Some Ca^{2+} channel agonists, e.g., Bay K 8644, have been demonstrated to shift the activation curve of $I_{Ca(L)}$ to negative potential by −10 to −15 mV, causing a left-ward shift of the I-V relationship of $I_{Ca(L)}$ (Markwardt and Nilius, 1988; McDonald et al., 1994), while Ca^{2+} channel antagonists do not cause such shift. DTZ323 blocked $I_{Ca(L)}$ at each voltage without shifting I-V relationships.

DTZ323 significantly shifted the steady-state inactivation curve towards a negative potential, suggesting that DTZ323 shifts Ca^{2+} channels to the inactivated state as is also found with diltiazem and dihydropyridines. Thus, at potentials more depolarized than −40 mV, DTZ323 produced tonic block. At negative potentials (V_h −90 ~ −50 mV), however, DTZ323 hardly produced tonic block. This suggests that the resting state has no significant affinity for the drug.

DTZ323 (10^{-6} M) suppressed $I_{Ca(L)}$ in a use-dependent manner at a holding potential ranging from −70 to −50 mV. The absolute use dependence suggests that DTZ323 gains access to either the open or the inactivated state of the channel. Development of the use-dependent block by DTZ323 at −70 mV was not simply dependent on the number of opening of channels, but was dependent on the frequency of test pulses (Fig. 5b). Under this condition, the duration of the test pulse (200 ms) is assumed to be long enough to cause voltage-dependent inactivation of channels. Thus these results indicate that DTZ323 decreases the rate of shift of the gating of Ca^{2+} channels from the inactivated state to the resting state.

The degree of the use-dependent block by DTZ323 depended on holding potentials, which can be explained by a voltage dependence of the rate of recovery from the block by DTZ323. If we assume that the drug does not have significant affinity for the channel in the resting state, and that the blockade is caused by the binding of the drug to the Ca^{2+} channel, the recovery from the block would reflect the dissociation of the drug from the channel. Thus, we compared the rate of recovery of $I_{Ca(L)}$ from the blockade by three drugs at different potentials. The results showed that the unblock of the blockade by DTZ323, diltiazem, and verapamil is slower at a depolarized membrane potential of around −50 mV than at −90 mV. At −50 mV, the rate of recovery was too slow to see any differences among the three drugs. These results indicate that the affinity of these drugs is higher at depolarized membrane potentials.

DTZ323 exhibited the fastest unblock followed by diltiazem and verapamil at voltages ranging from −90 mV to −60 mV (DTZ323 > diltiazem > verapamil). The little degree of block produced by DTZ323 at a holding potential of −90 mV (Fig. 5a) may be due to the fast dissociation of DTZ323 from Ca^{2+} channels (< 5 s), which allows the drug to dissociate from the channel before the next depolarizing test pulse is given at 0.2 Hz. On the other hand, the development of the use-dependent block of $I_{Ca(L)}$ by DTZ323 at −50 mV appears to be due to the slow dissociation of DTZ323 and accumulation of blockade.

The voltage dependence of the rate of recovery from the conditioned block may depend upon either the gating state of the Ca^{2+} channels or the intrinsic voltage dependence of the dissociation rate constant of the drug. Our finding that the blockade of $I_{Ca(L)}$ by DTZ323 took place in a state-dependent manner (no significant block at the resting state) and that DTZ323 delayed the shift of Ca^{2+} channels from the inactivated state to the resting state (frequency dependence) supports the idea that the voltage dependence of the use-dependent block by DTZ323 is caused by the state-dependent change of affinity of the drug for the channel.

The ionization state of the drugs may be an important factor to determine the dissociation rate of drugs. The pK_a values are 6.1 for DTZ323, 7.7 for diltiazem and 8.7 for verapamil. DTZ323 at physiological pH is supposed to be charged negatively, whereas diltiazem and verapamil are positively charged. The fraction of charged form of DTZ323 at physiological pH would be less than those of the other drugs, followed by diltiazem and verapamil. Our observation that unblock of diltiazem was faster than verapamil agrees with the idea that the drug in a positively charged form (verapamil) stays longer than the less charged form (diltiazem) (Kass et al., 1989). The minor fraction of the charged form of DTZ323 or negatively charged form at physiological pH might be related to its fast unblock.

We have previously shown that the negative inotropic effects of diltiazem and, to less extent, verapamil are potentiated by partial depolarization from −80 mV to −60 mV, whereas that of nifedipine is not affected (Okuyama et al., 1994). This was explained by the voltage dependence of the use-dependent block of $I_{Ca(L)}$ by verapamil as well as diltiazem. In this study, the rate of recovery from the block by DTZ323, diltiazem, and verapamil showed marked voltage dependence at −90 to −60 mV. This would be one of the important features for explaining the tissue selectivity of Ca^{2+} channel antagonists. The present results suggest that diltiazem, and presumably so does DTZ323, work more selectively on partially depolarized tissue such as the ischemic region in the heart than verapamil does (Okuyama et al., 1994).

Our results suggest that DTZ323 is selective to the L-type Ca^{2+} channel, the most potent among 1,5-benzothiazepine Ca^{2+} channel antagonists, and its blocking effect on $I_{Ca(L)}$ is strongly voltage dependent.

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