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# Functional interaction between benzothiazepine- and dihydropyridine binding sites of cardiac L-type $Ca^{2+}$ channels

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**Abstract**

We have previously shown, in a radioligand binding study with single ventricular myocytes, that benzothiazepine and dihydropyridine binding sites interact with each other. To further examine whether this interaction between the two binding sites is reflected in the function of L-type $Ca^{2+}$ channels, the blocking action of diltiazem, nitrendipine, and the combination of these two drugs on L-type $Ca^{2+}$ channel currents was investigated using baby hamster kidney cells expressing the $\alpha_{1C}$, $\alpha_2/\delta$, $\beta$ and $\gamma$ subunits of the $Ca^{2+}$ channel. The effects of diltiazem and nitrendipine were additive at room temperature but synergistic at 33°C. The use-dependent block by 3 $\mu$M of diltiazem was significantly enhanced from 28% to 68% by addition of 30 nM of nitrendipine, which by itself did not have a blocking effect. Thus, we conclude that benzothiazepine- and dihydropyridine binding sites interact and potentiate their blocking action on L-type $Ca^{2+}$ channels in a temperature-dependent manner. 

*Keywords:* $Ca^{2+}$ channel current; Diltiazem; Nitrendipine; Cardiac

## 1. Introduction

Voltage-dependent $Ca^{2+}$ channels are heteromultimeric proteins which consist of $\alpha_1$, $\alpha_2/\delta$, $\beta$ and $\gamma$ subunits (Hofmann et al., 1994). The L-type $Ca^{2+}$ channel in cardiovascular tissues appears to be composed of $\alpha_{1C}$, $\alpha_2/\delta$ and $\beta$ subunits and is one of the pharmacological targets of clinically useful $Ca^{2+}$ channel antagonists, e.g., dihydropyridines, phenylalkylamines and benzothiazepines. These three major drug groups have distinct binding sites on the $\alpha_1$ subunit of the L-type $Ca^{2+}$ channel and interact with each other allosterically (Catterall and Striessnig, 1992). In binding studies using membrane preparations, it has been shown that diltiazem and dihydropyridines inhibit each other's binding at low temperature, e.g., 4°C, but potentiate binding at 30–37°C, and that the extent of the potentiation varies depending on the dihydropyridine derivatives and experimental conditions such as membrane preparation and concentration of divalent cations (Glossmann et al., 1983, 1985). We have previously reported that diltiazem potentiates dihydropyridine binding in intact rat ventricular myocytes under physiological conditions (Kanda et al., 1997b). However, the pharmacological meaning of this interaction between the two drug-binding sites is not understood except for the possible synergistic negative inotropic effect (Garcia et al., 1986).

In spite of these findings, the pharmacological properties of dihydropyridines, phenylalkylamines and benzothiazepines are different from one another in respect to, for example, tissue selectivity. So far, extensive studies have been performed to classify these drug groups electrophysiologically. Dihydropyridines show almost exclusively tonic block while phenylalkylamines show solely use-dependent block and benzothiazepines are intermediate (Lee and Tsien, 1983). $Ca^{2+}$ channels, like $Na^+$ channels, are considered to have three distinct states, i.e., resting, activated and inactivated. It has been shown that the binding of $Ca^{2+}$ channel antagonists to the L-type $Ca^{2+}$ channel depends on the state of the channel and/or on the membrane potential. Bean (1984) has demonstrated electrophysiologically that nitrendipine binds to the channels in the inactivated state with an affinity of 1000 times higher than that for the channels in the resting state, assuming that current block is caused by 1:1 binding of nitrendipine to

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the channel. However, the precise mechanism of the modulation of the L-type $Ca^{2+}$ channel by $Ca^{2+}$ channel antagonist is still unclear.

The purpose of this study was to examine the functional interaction between benzothiazepine- and dihydropyridine binding sites on the L-type $Ca^{2+}$ channel. We found that nitrendipine potentiates the blocking action of diltiazem on $Ca^{2+}$ channel currents in a temperature-dependent manner. Together with our previous results derived from binding studies (Kanda et al., 1997b), the present results suggest that the interaction between benzothiazepine and dihydropyridine binding sites leads to potentiation of the blocking action on L-type $Ca^{2+}$ channel currents. Preliminary data of the present study have been presented in an abstract form (Kanda et al., 1997a).

## 2. Materials and methods

### 2.1. Cell culture

A baby hamster kidney (BHK) cell line stably expressing the rabbit cardiac $\alpha_{1C}$ subunit and rabbit skeletal $\alpha_2/\delta$, $\beta_1$, and $\gamma$ subunits (BHKC12) was provided by Eisai Tsukuba Research Laboratories. Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum, 30 $\mu$g ml$^{-1}$ streptomycin and 30 units ml$^{-1}$ penicillin and selected by 600 $\mu$g ml$^{-1}$ geneticin and 250 nM methotrexate as previously described (Hirano et al., 1996).

### 2.2. Electrophysiological measurement

The BHKC12 cells were plated on plastic coverslips (Sumilon, Tokyo, Japan) and cultured for 1–3 days before electrophysiological measurements. Whole-cell $Ca^{2+}$ channel currents were measured by the whole-cell patch-clamp technique (Hamill et al., 1981). The recording chamber was perfused continuously with Tyrode solution (NaCl 137 mM, KCl 5.4 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, HEPES 10 mM, glucose 10 mM, pH 7.4 with NaOH). The resistance of the heat-polished microelectrodes was 1–4 M$\Omega$ when filled with the internal solution (CsCl 110 mM, TEA–Cl 30 mM, HEPES 10 mM, cAMP 0.2 mM, ATP–Mg 5 mM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid 10 mM, $CaCl_2$ 2 mM, NaCl 1 mM, pH 7.3 with CsOH). Whole-cell patch-clamp recordings were performed after the $Ca^{2+}$ channel currents had become stable. The current–voltage relationships were measured to confirm the accuracy of the clamp. $Ca^{2+}$ channel currents were recorded at room temperature (about 23°C) or at a higher temperature (about 33°C). The membrane capacitance of the cells in the present study was $53 \pm 2$ pF ($n = 120$). Leak currents were subtracted by applying a hyperpolarized potential (P/4 protocol) except for the steady-state inactivation.

The $Ca^{2+}$ channel currents were filtered at 5 kHz and digitized at 2.5 kHz, using an Axopatch 1D amplifier (Axon Instruments, Burlingame, CA, USA). Current stimulation and data acquisition were performed by using pCLAMP6 software (Axon Instruments) on an IBM AT personal computer.

### 2.3. Data analysis

The peak amplitude of recorded currents was analyzed by using clampfit on pCLAMP6 software. All parameters of the subsequent analysis were calculated by computer fitting (GraphPad, Inplot). The steady-state inactivation curves were fitted by the Boltzman equation: $I/I_{max} = 1/\{1 + \exp[-(V - V_{0.5})/k]\}$ where $V_{0.5}$ is the mid-potential for inactivation and $k$ is the slope of the curve. The $IC_{50}$ values for block of $Ca^{2+}$ channel currents by diltiazem and nitrendipine were calculated assuming 1:1 binding.

Results are expressed as means $\pm$ S.E.M. Statistical significance was assessed with Student–Welch's *t*-test for simple comparisons or with Bonferroni's multiple *t*-test. Differences at $P < 0.05$ were considered to be significant.

### 2.4. Chemicals

Diltiazem was a generous gift from Tanabe Seiyaku (Saitama, Japan). Nitrendipine was a gift from Yoshitomi Pharmaceutical (Osaka, Japan). All other materials were of reagent grade quality and obtained from standard sources.

## 3. Results

### 3.1. Characteristics of the whole-cell $Ca^{2+}$ channel currents expressed in BHKC12 cells

We first characterized the whole-cell $Ca^{2+}$ channel currents expressed in BHKC12 cells. We included 0.2 mM cAMP in the internal solution to enhance and stabilize $Ca^{2+}$ channel currents, because it has been shown that the open probability of $Ca^{2+}$ channels of BHKC12 is increased by bath application of 8Br-cAMP (Hirano et al., 1996). When depolarizing pulses to 0 mV for 100 ms applied from the holding potential at $-100$ mV, we observed typical L-type $Ca^{2+}$ channel currents which activated and inactivated rapidly and were sensitive to dihydropyridines and $Cd^{2+}$.

Fig. 1 shows a typical example of the current–voltage relationships and the steady-state inactivation curve at room temperature. The threshold voltage for the activation of the $Ca^{2+}$ channel current was around $-40$ mV and the peak current was elicited by the test potential to around 0 mV, which is the typical for L-type $Ca^{2+}$ channel currents. In contrast to the activation, the steady-state inactivation was remarkably different from that of native cardiac $Ca^{2+}$

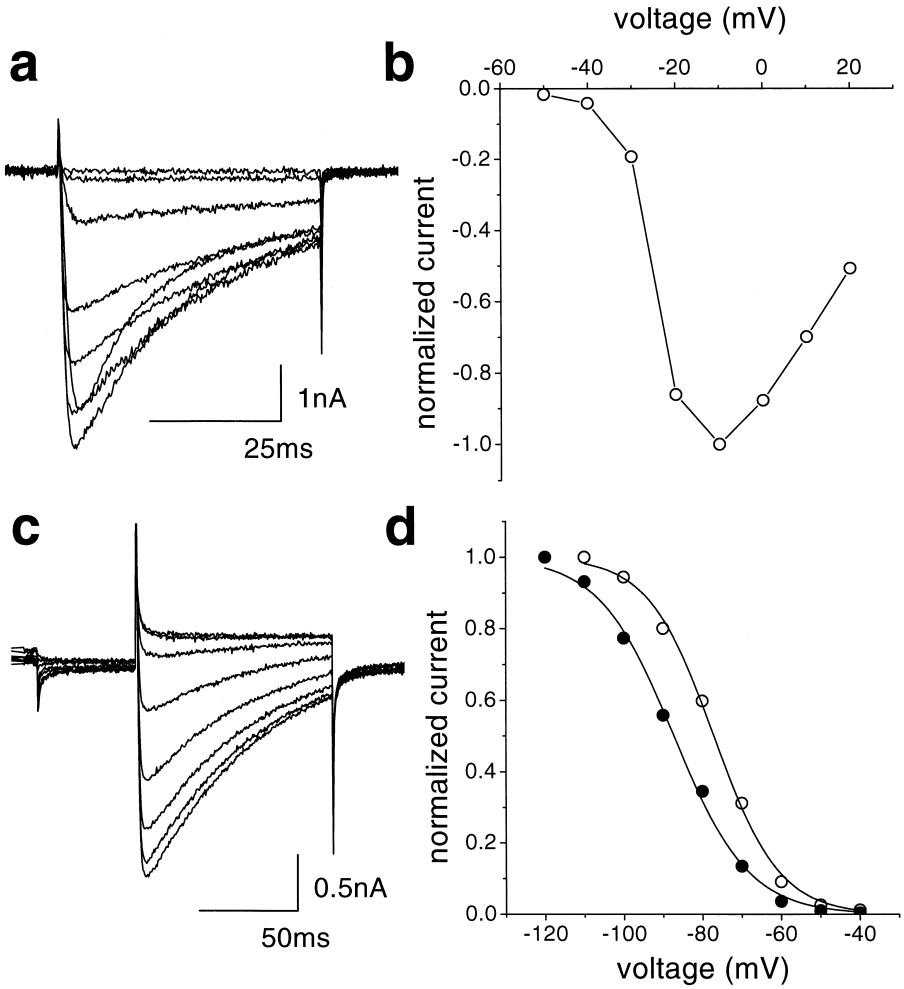


Fig. 1. Typical current–voltage relationship (a, b) and steady-state inactivation curve (c, d) for the $Ca^{2+}$ channel stably expressed in BHK cell at room temperature. (a) Currents were elicited from a holding potential of −100 mV. Depolarizing pulses of increasing voltage (10 mV increment) for 100 ms were applied at 0.083 Hz. (b) Plot of the normalized current–voltage relationships obtained in (a). (c) Steady-state inactivation was measured by applying prepulses to voltages ranging from −110 to −20 mV for 5 s, followed by a test pulse to 0 mV for 100 ms. Holding potential was −100 mV and pulses were applied at 0.033 Hz. (d) Steady-state inactivation curves measured in the absence and presence of 10 nM of nitrendipine. A BHKC12 cell was perfused with drug-free solution and steady-state inactivation was measured shown as in (c) (open circle), then 10 nM of nitrendipine was applied and steady-state inactivation was measured again (closed circle). Peak currents during test pulses were normalized by the maximal value. Data were fitted to the Boltzman equation as described in Section 2. $V_{0.5} = -77.1$ mV and $k = 8.3$ mV in the absence of drug, and $V_{0.5} = -87.3$ mV and $k = 9.5$ mV in the presence of 10 nM of nitrendipine.

channels. The $V_{0.5}$ of the steady-state inactivation curve was $-70.0 \pm 2.1$ mV and $k$ was $9.20 \pm 0.38$ mV ($n = 15$) at room temperature. This $V_{0.5}$ value was shifted in the hyperpolarizing direction by about 50 mV compared to that of the native cardiac L-type $Ca^{2+}$ channel current recorded from guinea-pig ventricular myocytes. These values were not different at a higher temperature (33°C, $V_{0.5} = -71.7 \pm 2.0$ mV, $k = -8.68 \pm 0.42$ mV, $n = 8$). When we compared the steady-state inactivation in the absence and the presence of 10 nM of nitrendipine, as shown in Fig. 1d, $V_{0.5}$ was shifted in a hyperpolarizing direction by $10.9 \pm 0.6$ mV ($P < 0.001$ vs. time control group $3.79 \pm 1.00$ mV, $n = 8$) and $k$ was decreased slightly by $1.42 \pm 0.48$ mV ($P < 0.05$ vs. time control group $0.22 \pm 0.26$ mV, $n = 8$). Thus, the holding potential was set at −100 mV throughout this study.

We also examined the recovery of $Ca^{2+}$ channel currents with double-pulse protocol. A prepulse to 0 mV for 100 ms was followed by a test pulse to 0 mV for 100 ms separated by various recovery times between 50 ms and 2 s at −100 mV. The kinetics of recovery consisted of two components. Time constants and the proportion of fast and slow components were $15.4 \pm 4.0$ ms and $446 \pm 99$ ms, and $0.624 \pm 0.036$ and $0.317 \pm 0.029$, respectively ($n = 7$). Thus, the pulse frequency was set at 0.1 Hz in the following experiments.

### *3.2. Effects of nitrendipine, diltiazem, and the combination of both drugs at room temperature*

We examined the concentration–response relationships for nitrendipine and diltiazem on $Ca^{2+}$ channel currents in

BHKC12 cells. Nitrendipine or diltiazem at concentrations ranging between 1 nM and 10 μM was cumulatively applied and the $IC_{50}$ values were obtained by fitting the concentration–response curve to the Hill equation. The $IC_{50}$ values of nitrendipine and diltiazem were 96 ± 35 nM ($n = 6$) and 2.4 ± 1.1 μM ($n = 7$), respectively. Although some $Ca^{2+}$ channel antagonists have been reported to accelerate the decay of $Ca^{2+}$ channel currents, neither diltiazem, nitrendipine, nor the combination of two drugs accelerated the decay of $Ca^{2+}$ channel currents elicited by 100 ms test pulse. Fig. 2 shows typical traces recorded at room temperature. In order to examine the potentiation of the use-dependent blocking action of diltiazem by nitrendipine, we set the concentration of nitrendipine at 30 nM to elicit minimum block of $Ca^{2+}$ channel currents and that of diltiazem at 3 μM to give moderate block in the following experiment.

Because dihydropyridine causes a clear tonic block of the $Ca^{2+}$ channel current and diltiazem causes a clear use-dependent block, we used the following protocol to distinguish the two types of blockade. $Ca^{2+}$ channel currents were elicited at 0.1 Hz for at least 1 min to ensure current stability, then the stimulation was stopped, and drugs were perfused in the absence of stimulation. Following drug application for 3 min, stimulation was resumed and currents were measured for 5 min. The tonic block was measured as the difference between the $Ca^{2+}$ channel amplitude elicited by the last pulse before drug application and the first pulse recorded after 3 min of drug application. The use-dependent block was measured as the block developed during the 5 min of stimulation in the presence of drugs.

Fig. 3 shows the time-course of the block of $Ca^{2+}$ channel currents produced by each drug. The time point of

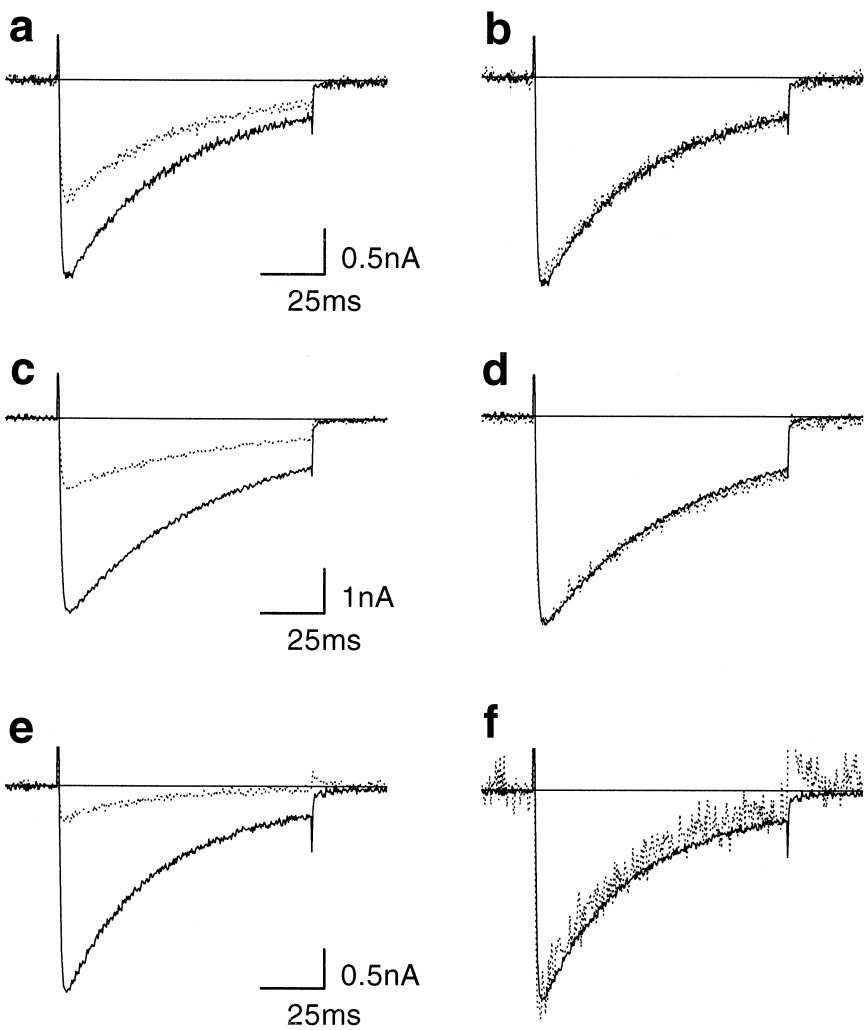


Fig. 2. Effects of 30 nM of nitrendipine (a, b), 3 μM of diltiazem (c, d) and the combination of both drugs (e, f) on the kinetics of L-type $Ca^{2+}$ channel current at room temperature. (a, c, e) Typical current traces measured during application of nitrendipine, diltiazem, and the combination of both drugs, respectively. Solid line indicates the control current and the dotted line indicates the current in the presence of each drug. (b, d, e) The normalized current traces of (a), (c) and (e), respectively. Horizontal line indicates zero current level.

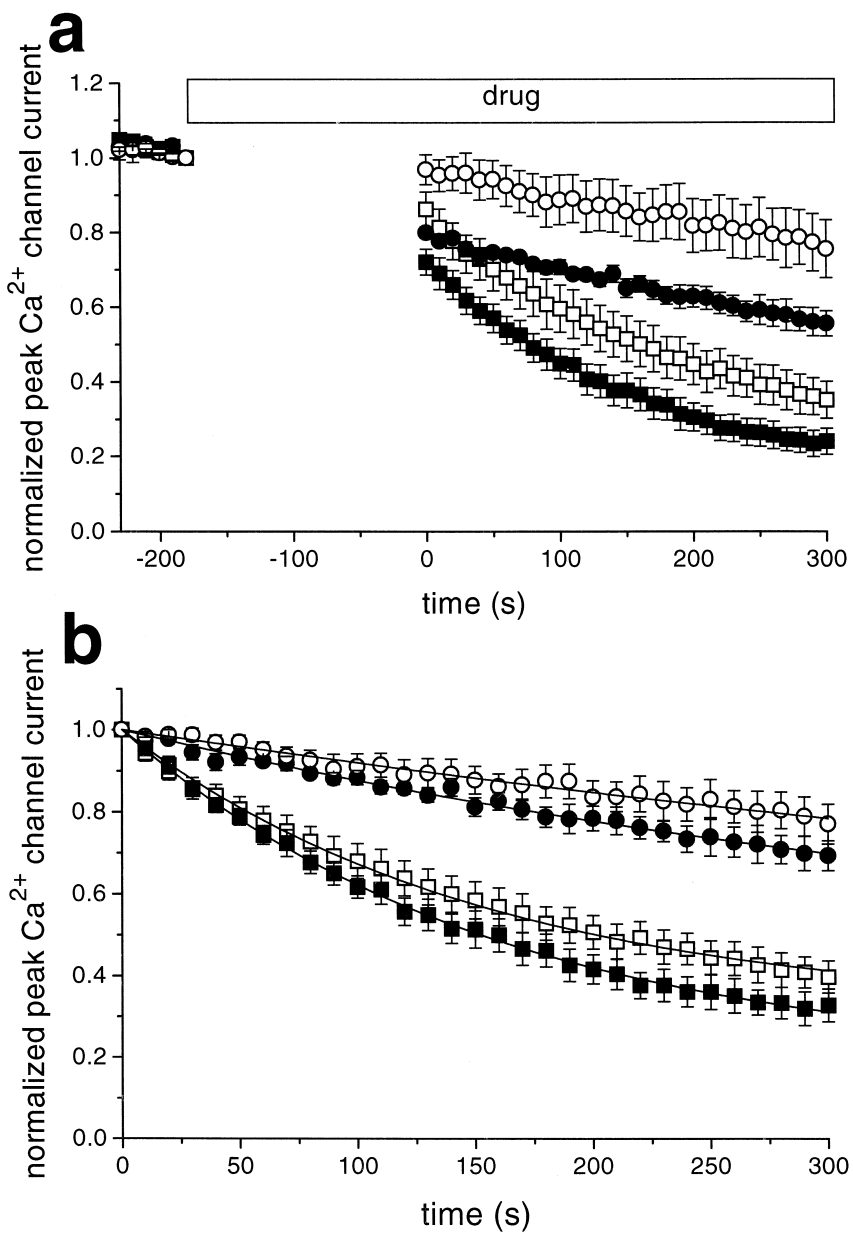


Fig. 3. Time-course of blockade of $Ca^{2+}$ channel currents by 30 nM of nitrendipine (●), 3 μM of diltiazem (□), or the combination of both drugs (■) at room temperature. Currents were elicited by applying test pulses to 0 mV for 100 ms from a holding potential of −100 mV at 0.1 Hz. Drugs were applied for 3 min without stimulation and then currents were recorded for 5 min in the presence of drugs. Time control is also shown (○). Each point represents mean ± S.E.M. (a) Peak amplitude of the $Ca^{2+}$ channel currents was normalized relative to that recorded just before drug application. (b) Development of the use-dependent block of $Ca^{2+}$ channel currents in the presence of drugs. Peak amplitude of $Ca^{2+}$ channel currents was normalized relative to that elicited by the first pulse after drug application.

resumption of stimulation was set at 0 s and the peak amplitude of $Ca^{2+}$ channel currents was normalized relative to that of currents recorded just before drug application (Fig. 3a). There was little decrease in the current amplitude in the time control group measured in the absence of drugs (3.1 ± 4.0%). The tonic suppression of $Ca^{2+}$ channel currents after 3 min of drug application was 20.0 ± 1.3% with nitrendipine at 30 nM, 13.8 ± 4.7% with diltiazem at 3 μM, and 28.0 ± 3.5% with the combination of both drugs. Nitrendipine and the combination of nitrendipine and diltiazem caused significant tonic block ($P < 0.05$ vs. control). The tonic block of $Ca^{2+}$ channel current, estimated as percentage of the time control, was 17% with nitrendipine, 11% with diltiazem, and 26% with the combination of both drugs, thus indicating that the tonic block of the combination of two drugs was additive.

To analyze the use-dependent block, the peak amplitudes of $Ca^{2+}$ channel currents were normalized relative to that elicited by the first pulse after drug application (Fig. 3b). The time course of the change in peak current ampli-

tude of the time control and nitrendipine groups roughly overlapped, indicating that nitrendipine at 30 nM caused clear tonic block but hardly caused use-dependent block. Diltiazem at 3 μM caused prominent use-dependent block with little tonic block. The block caused by the combination of these two drugs was obviously larger than that of the respective drug alone and both the tonic block and the use-dependent block were noticeable. The use-dependent block of $Ca^{2+}$ channel current at the steady-state (at 300 s), estimated as percentage of the time control, was 10% with nitrendipine, 48% with diltiazem, and 58% with the combination of both drugs. The overall effect of the combination of the two drugs was similar to the sum of the block produced by the respective drugs. Thus, nitrendipine did not potentiate the blocking action of diltiazem on $Ca^{2+}$ channel currents when measured at room temperature.

### 3.3. Effects of nitrendipine, diltiazem, and combination of both drugs at higher temperature

Radioligand binding studies have shown that the interaction of diltiazem and dihydropyridine sites depends on the temperature (Glossmann et al., 1983). Thus, we per-

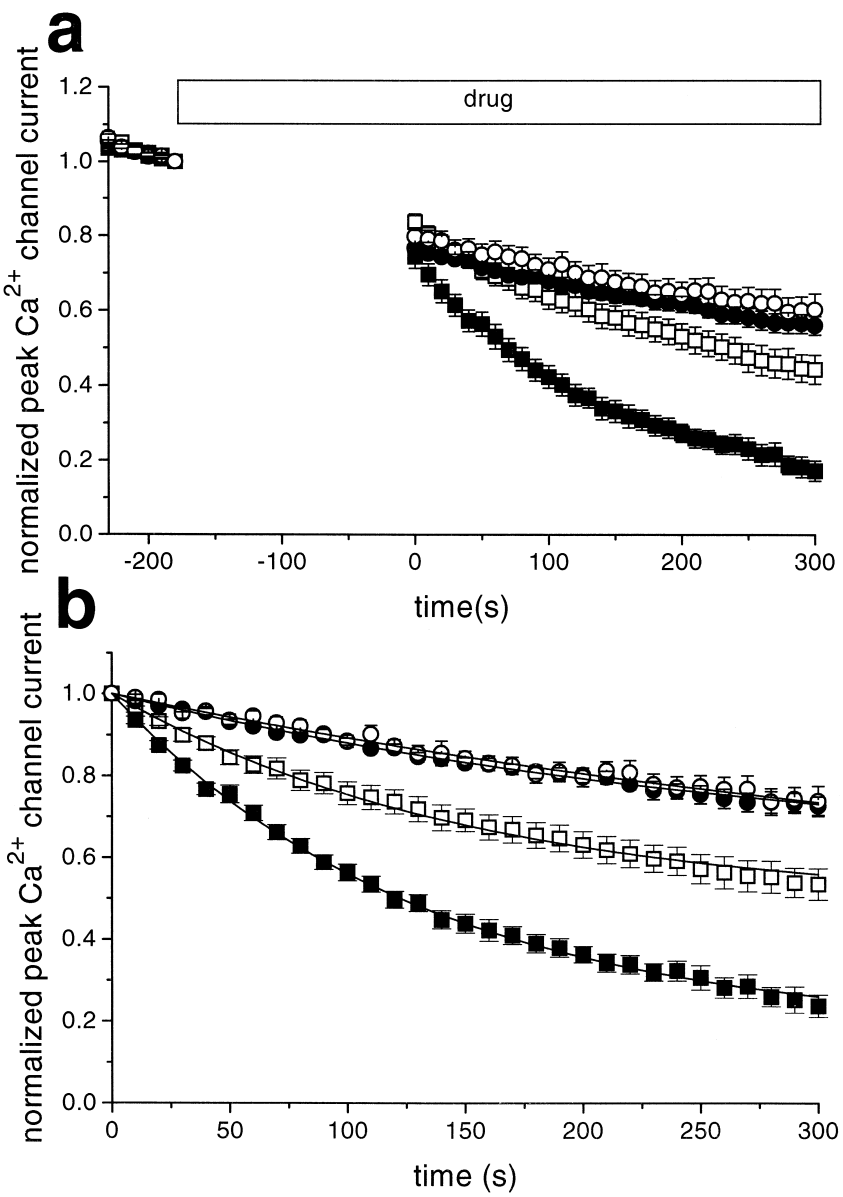


Fig. 4. Time-course of blockade of $Ca^{2+}$ channel currents by 30 nM of nitrendipine (●), 3 μM of diltiazem (□), or the combination of both drugs (■) at 33°C. The pulse protocol was identical to that described in the legend to Fig. 3. Control measurement in the absence of drugs is also shown (○). Each point represents mean ± S.E.M. (a) Peak amplitude of $Ca^{2+}$ channel currents was normalized relative to that recorded just before drug application. (b) Development of the use-dependent block of $Ca^{2+}$ channel currents in the presence of drugs. Peak amplitude of $Ca^{2+}$ channel currents were normalized relative to that elicited by the first pulse after the drug application.

formed the same experiments at 33°C. The peak amplitude of $Ca^{2+}$ channel currents tended to be greater than that measured at room temperature, but we observed obvious run-down of the current even in the absence of drugs. This run-down made the estimation of the tonic block component more difficult. Fig. 4a shows the time-course of the peak current amplitude normalized relative to that for the last pulse recorded just before drug application. Compared to room temperature, the block of $Ca^{2+}$ channel currents produced by diltiazem or nitrendipine by itself was apparently decreased, which is likely to be due to the weak temperature dependence of the binding affinity of these drugs for the $Ca^{2+}$ channel. At this temperature, the peak amplitude of the $Ca^{2+}$ channel current of the time control group was decreased by $20.2 \pm 2.3\%$ during vehicle application and this value was significantly greater than that obtained at room temperature ($P < 0.01$), which indicates that run-down of $Ca^{2+}$ channel currents was promoted at higher temperature. The decrease of the current amplitude during 3 min of drug application was $23.3 \pm 1.4\%$ with nitrendipine at 30 nM, $16.4 \pm 2.2\%$ with diltiazem at 3 μM, and $25.6 \pm 3.2\%$ with both drugs. Neither nitrendipine, diltiazem, nor the combination of the drugs caused significant tonic block.

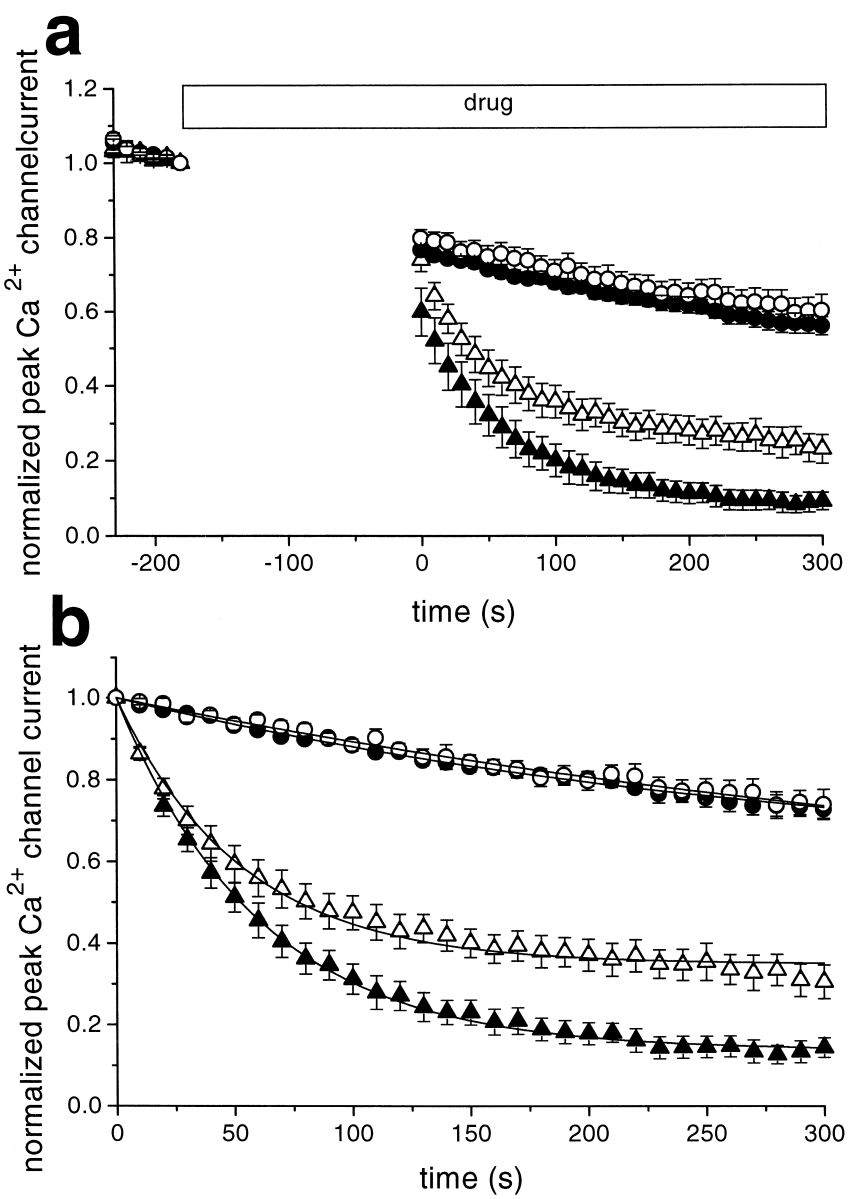


Fig. 5. Time-course of blockade of $Ca^{2+}$ current by 10 μM diltiazem (△) or the combination of 30 nM of nitrendipine and 10 μM of diltiazem (▲) at 33°C. The pulse protocol was identical to that described in the legend to Fig. 3. Control (○) and 30 nM of nitrendipine (●) are the same as in Fig. 4. Each point represents mean ± S.E.M. (a) Peak amplitude of $Ca^{2+}$ channel currents was normalized relative to that recorded just before drug application. (b) Development of the use-dependent block of $Ca^{2+}$ channel currents in the presence of drugs. Peak amplitude of $Ca^{2+}$ channel currents was normalized relative to that elicited by the first pulse after drug application.

Table 1
Kinetic parameters of the use-dependent block

| | Plateau | $\tau$ (s) | $n$ |
|---|---|---|---|
| Room temperature | | | |
| Diltiazem $3 \times 10^{-6}$ M | $0.280 \pm 0.069$ | $161 \pm 32$ | 5 |
| Nitrendipine $3 \times 10^{-8}$ M + diltiazem $3 \times 10^{-6}$M | $0.159 \pm 0.051$ | $173 \pm 20$ | 6 |
| 33°C | | | |
| Diltiazem $3 \times 10^{-6}$ M | $0.425 \pm 0.051$ | $176 \pm 26$ | 9 |
| Nitrendipine $3 \times 10^{-8}$ M + diltiazem $3 \times 10^{-6}$ M | $0.168 \pm 0.026$* | $137 \pm 12$ | 11 |
| Diltiazem $10^{-5}$ M | $0.334 \pm 0.036$ | $60.0 \pm 7.7$** | 7 |
| Nitrendipine $3 \times 10^{-8}$ M + diltiazem $10^{-5}$ M | $0.130 \pm 0.020$* | $64.0 \pm 6.6$** | 8 |

Parameters were calculated by fitting the data of Fig. 3b, Fig. 4b and Fig. 5b to the following equation: $I = (1 - \text{plateau})\exp(-t/\tau) + \text{plateau}$. $I$ is the relative peak amplitude of $Ca^{2+}$ channel current normalized by the first pulse after the drug application. $\tau$ is the time constant and plateau is the normalized $Ca^{2+}$ channel current measured when the use-dependent block reached steady state. Each value represents mean ± S.E.M.
* $P < 0.01$ vs. diltiazem alone.
** $P < 0.05$ vs. lower concentration of diltiazem.

Nitrendipine 30 nM did not attenuate $Ca^{2+}$ channel currents during stimulation at 0.1 Hz, while diltiazem at 3 μM suppressed the currents modestly in a use-dependent manner. These effects became clearer when the peak current amplitude was normalized to that of the first pulse after drug application (Fig. 4b). Surprisingly, the combination of nitrendipine and diltiazem markedly inhibited the L-type $Ca^{2+}$ channel current. The use-dependent block of $Ca^{2+}$ channel current at the steady-state (at 300 s), estimated as percentage of the time control, was 1.6% with nitrendipine at 30 nM, 28% with diltiazem at 3 μM, and 68% with the combination of both drugs. In contrast to the results obtained at room temperature, the use-dependent block produced by diltiazem was apparently enhanced by the addition of 30 nM of nitrendipine.

To examine whether this enhancement of the block of $Ca^{2+}$ channel currents is caused by an increase in the affinity of $Ca^{2+}$ channels for diltiazem, the experiment was repeated with a higher concentration of diltiazem (10 μM) (Fig. 5). In this case, the tonic suppression of the $Ca^{2+}$ channel current was $26.0 \pm 3.1\%$ with diltiazem at 10 μM and $40.2 \pm 6.5\%$ with the combination of drugs. The tonic block of $Ca^{2+}$ channel current, estimated as percentage of the time control, was 4% with nitrendipine at 30 nM, 7.3% with diltiazem at 10 μM, and 25% with the combination of both drugs. The tonic block produced by nitrendipine was significantly augmented by the addition of diltiazem at 10 μM.

Diltiazem at 10 μM by itself inhibited $Ca^{2+}$ channel currents in a use-dependent manner without causing tonic block. When diltiazem at 10 μM was combined with nitrendipine at 30 nM, the use-dependent block of the current was greater. The use-dependent block of $Ca^{2+}$ channel current at the steady-state (at 300 s), estimated as percentage of the time control, was 59% with diltiazem at 10 μM and 81% with the combination of nitrendipine (30 nM) and diltiazem (10 μM). Thus, the use-dependent block produced by diltiazem at 10 μM was also enhanced by the addition of 30 nM of nitrendipine.

### 3.4. Analysis of the use-dependent block

For further analysis of the use-dependent block, the time course of the change in the relative peak $Ca^{2+}$ channel current amplitude was fitted to a model of mono-exponential decay, which gave the best fit. The calculated time constants of development of the use-dependent block ($\tau$) and the relative amplitude of the use-dependent block component (plateau) are summarized in Table 1. At room temperature, neither of these parameters was statistically different for diltiazem alone and for the combination of diltiazem and nitrendipine. These results indicate that the use-dependent block produced by these two groups was nearly identical. Taking the results of Fig. 3 into account, it is concluded that diltiazem and nitrendipine exerted their effect independently at room temperature.

In contrast, the steady-state of the use-dependent block of $Ca^{2+}$ channel currents produced by the combination of diltiazem at 3 μM and nitrendipine at 30 nM was significantly greater than that caused by diltiazem alone at 33°C ($P < 0.01$). The time constants obtained in the presence of diltiazem at 3 μM or the combination of diltiazem at 3 μM and nitrendipine at 30 nM were not different at either temperature. These effects were in contrast to the effect of a higher concentration of diltiazem (10 μM), which increased both the steady-state block and the time constant of development of the block ($P < 0.05$). Thus, at 33°C, the effect of diltiazem on $Ca^{2+}$ channel currents appeared to be potentiated by the addition of nitrendipine through the allosteric interaction between the nitrendipine- and diltiazem binding sites.

## 4. Discussion

We have previously reported that the interaction between the binding sites for benzothiazepine and dihydropyridine on functional $Ca^{2+}$ channels occurs under physiological conditions in freshly isolated rat ventricular my-

ocytes (Kanda et al., 1997b). In this study, we examined whether this interaction is reflected by the function of the L-type $Ca^{2+}$ channels, using BHKC12 cells which stably express cardiac L-type $Ca^{2+}$ channels. We found that the block of the L-type $Ca^{2+}$ channel current caused by diltiazem at 3 μM and nitrendipine 30 nM was additive at room temperature, but synergistic at 33°C. The synergistic increase in the block of the L-type $Ca^{2+}$ channel current amplitude produced by diltiazem and nitrendipine, and the temperature dependence of the effect agreed well with the well-known potentiation of diltiazem and dihydropyridine binding in radioligand binding studies (Glossmann et al., 1985; Glossmann and Striessnig, 1990).

Although the potentiation between diltiazem and dihydropyridines is well-known in binding studies, the mechanism has not been clarified. We analyzed the use-dependent block of $Ca^{2+}$ channel currents to elucidate the underlying mechanism and tried to interpret these results using the model of Timin and Hering (1992) with some modifications. In addition to their assumptions, we assumed that the use-dependent block of $Ca^{2+}$ channel currents is caused solely by diltiazem, and that diltiazem associates with the channel at a constant rate when the channels are depolarized and dissociates from the channel at a constant rate during the resting state. When the use-dependent block of $Ca^{2+}$ channel currents caused by the $n$th stimulation pulse ($b_n$) is plotted against the pulse number ($n$), $b_n$ is expressed by the following equation:

$$b_n = b_{ss}(1 - e^{-n\lambda}).$$

When the rate constant for the association of diltiazem to the depolarized channel is $k_1$ $M^{-1}$ $s^{-1}$, the rate constant for the dissociation of diltiazem from the resting channel is $k_{-1}$ $s^{-1}$, depolarizing pulse duration is $t_d$ s, stimulation frequency is $1/t_r$ Hz, and the concentration of diltiazem is $D$ M, $\lambda$ and $b_{ss}$ are expressed by the following equations:

$$\lambda = k_1 \cdot D \cdot t_d + k_{-1} \cdot t_r,$$

$$b_{ss} = (e^{-k-1 \cdot tr} - e^{-\lambda})/(1 - e^{-\lambda}).$$

With a stimulation frequency of 0.1 Hz, the time constant ($\tau$) and the steady-state block of the use-dependent block (plateau) shown in Table 1 are equivalent to $10 \cdot \lambda$ and $1\text{-}b_{ss}$, respectively. As is expected from the model, the use-dependent block developed faster and the steady-state block was greater with diltiazem at 10 μM than at 3 μM. In contrast to this, the time constant of development of the use-dependent block induced by the combination of diltiazem and nitrendipine was similar to that induced by diltiazem alone, although the steady-state block was increased (Table 1). According to the model, these results indicate that nitrendipine increases the association rate and decreases the dissociation rate of diltiazem to and from its own binding site, and suggest that the nature of this interaction between diltiazem and dihydropyridine binding sites is allosteric rather than steric as proposed by Brauns et al. (1997) who used a fluorescent benzazepin analogue as a ligand for the benzothiazepine binding site. These findings are in agreement with previous reports which have shown that dihydropyridine modulates both the association and dissociation rates of diltiazem and vice versa in radioligand binding studies (Garcia et al., 1986; Ikeda et al., 1991). Such a modulation of the binding kinetics of diltiazem by nitrendipine may explain the potentiated use-dependent block of the $Ca^{2+}$ channel current.

Many studies have been performed to determine the localization of the binding sites for dihydropyridine, phenylalkylamine, and benzothiazepine by using molecular biological techniques (Hockerman et al., 1997). It has been shown that the major molecular determinants of dihydropyridine binding are segment (6) of motifs III and IV and that the major determinant of phenylalkylamine and benzothiazepine binding is segment (6) of motif IV. These findings indicate that the molecular determinants of dihydropyridine and diltiazem binding sites are distinct but partially overlapping (Hering et al., 1996). Thus, because of the proximity of their binding sites, the potentiation of the blocking effect of diltiazem on $Ca^{2+}$ channel currents by nitrendipine observed in this study may be induced by conformational change of the $Ca^{2+}$ channel and of the environment around the drug-binding sites caused by binding of another drug. Alternatively, it has been postulated that diltiazem increases the inactivated state of $Ca^{2+}$ channels to which dihydropyridines preferentially bind (Glossmann et al., 1985), and that the use-dependent block by diltiazem is steeply voltage-dependent over the physiological voltage range (Okuyama et al., 1994). Because the membrane voltage was clamped in this study, voltage-dependent inactivation of the $Ca^{2+}$ channel was limited. Therefore, the binding of diltiazem or nitrendipine to the $Ca^{2+}$ channel may cause a conformational change which mimics the voltage-dependent inactivated state of the $Ca^{2+}$ channel and thus increases the affinity for the other drug.

One surprising finding in this study is the temperature dependence of the interaction between diltiazem and nitrendipine binding sites. It has been shown that temperature affects the activation, inactivation, and amplitude of the $Ca^{2+}$ channel current with a high $Q_{10}$ (Allen, 1996). The potency of diltiazem and nitrendipine alone to cause $Ca^{2+}$ channel blockade decreased at higher temperature, which is consistent with the slight decrease in affinity of PN200-110 (isladipine) at higher temperature found in radioligand binding studies (Ikeda et al., 1991; Zheng et al., 1991). In spite of this, the time constant of the development of the use-dependent block produced by 3 μM of diltiazem at room temperature was the same as that at 33°C. According to the model described above, these results indicate that the association rate of diltiazem decreases and the dissociation rate of diltiazem increases at higher temperature. As to dihydropyridines, it has been shown that the association rate constant of [$^3$H]PN200-110 (isladipine) increases slightly and the dissociation rate constant increases remarkably between 4°C and 37°C

(Zheng et al., 1991). Because the L-type $Ca^{2+}$ channels were exogenously expressed and a high concentration of cAMP (0.2 mM) was included in the internal solution in this study, the involvement of phosphorylation can be ruled out. Thus, a conformational change of the channel by itself seems to be responsible for the temperature dependence of the block. The degree of conformational change(s) of the $Ca^{2+}$ channel induced by the binding of each drug may be different depending on the temperature. Further efforts are needed to clarify the molecular mechanisms underlying this temperature dependence.

In this study, we used BHKC12 cells expressing cardiac $\alpha_{1C}$ subunit and skeletal muscle $\alpha_2/\delta$, $\beta_1$, and $\gamma$ subunits (Hirano et al., 1996). Although the $\gamma$ subunit is expressed specifically in skeletal muscle, $Ca^{2+}$ channels expressed in BHKC12 cells exhibit many properties characteristic of cardiac L-type $Ca^{2+}$ channels. Skeletal muscle L-type $Ca^{2+}$ channels show a characteristic slow activation rate and cardiac L-type $Ca^{2+}$ channels activate faster with a time constant of 6–10 ms (McDonald et al., 1994). $Ca^{2+}$ channels expressed in BHKC12 cells activated rapidly in response to a test potential to 0 mV from a holding potential of −100 mV and inactivated rapidly (Fig. 1). The threshold voltage for activation (−50 mV) and the voltage which gave the peak current amplitude (−10 mV) were also consistent with those of native cardiac L-type $Ca^{2+}$ channels (McDonald et al., 1994). Furthermore, pharmacological properties such as sensitivity to $Ca^{2+}$ channel antagonists and $Cd^{2+}$ were similar to those of the native channel. The only difference between $Ca^{2+}$ channels expressed in BHKC12 cells and native $Ca^{2+}$ channels was the steady-state inactivation. The $V_{0.5}$ of the steady-state inactivation curve was shifted in a hyperpolarizing direction (50 mV, Fig. 1). A recent study has shown that the skeletal muscle $\gamma$ subunit can interact with the cardiac $\alpha_{1C}$ subunit, and that this subunit, when expressed in addition to $\alpha_{1C}$, $\alpha_2/\delta$ and $\beta$ subunits, can accelerate inactivation and thus cause the hyperpolarizing shift of the steady-state inactivation curve by 20 mV without affecting the activation kinetics (Eberst et al., 1997). We confirmed that 10 nM of nitrendipine shifted the steady-state inactivation curve in a more hyperpolarizing direction. Thus, the abnormality of the steady-state inactivation cannot be due to contamination of the control bath solution with the residual nitrendipine, but must be an intrinsic property of the $Ca^{2+}$ channel expressed in BHKC12 cells. In addition, it has been shown with [$^3$H]PN200-110 (isladipine) that the minimum subunit composition for complete reconstitution of the pharmacological properties of cardiac dihydropyridine-sensitive L-type $Ca^{2+}$ channels requires the $\alpha_{1C}$, $\alpha_2/\delta$, and $\beta$ subunits, and that further coexpression of the $\gamma$ subunit dose not affect on the pharmacological properties (Wei et al., 1996). The electrophysiological and pharmacological characteristics observed in the present study of $Ca^{2+}$ channels expressed in BHKC12 cells are consistent with these previous reports.

In this study, we showed that diltiazem and nitrendipine interact with each other to affect the function of cardiac L-type $Ca^{2+}$ channels in a temperature-dependent manner and potentiate their block of $Ca^{2+}$ channel currents at 33°C. These results are consistent with our previous report that diltiazem modulates dihydropyridine binding in intact cardiac ventricular myocytes under physiological conditions (Kanda et al., 1997b). We conclude that the benzothiazepine binding site interacts with the dihydropyridine binding site in a temperature-dependent manner, and that this interaction is reflected by the potentiated block of the L-type channel current.

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