Mechanisms of long-lasting effects of benidipine on Ca current in guinea-pig ventricular cells

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1 Benidipine (KW-3049), a new derivative of 1,4-dihydropyridine (DHP), showed dose-dependent inhibition of Ca current (I_{Ca}) which was elicited by depolarization from -40 mV to $+10$ mV at 0.2 Hz in single cardiac cells isolated from guinea-pig ventricle under whole cell voltage clamp. Half inhibition doses (IC_{50}) of benidipine and nifedipine for the peak I_{Ca} at $+10$ mV were 2.7 nM and 63.1 nM, respectively.

2 A change in holding potential from -40 to -75 mV partially removed the block induced by both 10 nM benidipine and 100 nM nifedipine. The block of I_{Ca} by benidipine strongly depended upon holding potentials as did that induced by nifedipine.

3 The effect of 100 nM nifedipine was mostly removed when the cells were kept quiescent at holding potentials negative to -75 mV for 5 min after withdrawal of nifedipine. In contrast, hyperpolarization for several minutes did not significantly accelerate the removal of benidipine-induced block after withdrawal of the drug. Effects of 10 nM benidipine could not be washed out for up to 30 min regardless of the holding potentials.

4 It is suggested that the dissociation of benidipine from the DHP binding site, like that of nifedipine, is greatly accelerated by hyperpolarization. Benidipine but not nifedipine may have an additional interaction with the channel or lipid membrane and cannot be washed away even after the dissociation. Alternatively, the dissociation of benidipine from the DHP binding site may be too slow to occur substantially during the limited period of hyperpolarization in the present study (<30 min). In that case, however, the Ca channel bound by benidipine must become unblocked and available to be opened during the hyperpolarization.

Introduction

Benidipine is a dihydropyridine derivative which has a strong and long-lasting antihypertensive effect (Karasawa *et al.*, 1988a) as well as an antianginal effect (Karasawa *et al.*, 1988b). The inhibitory effects of benidipine on ^{45}Ca -influx (Karasawa & Kubo, 1988) and Ba^{2+} current (Terada *et al.*, 1987) have been demonstrated in canine coronary artery and single smooth muscle cells isolated from rabbit mesenteric artery, respectively. The rate of rise in slow action potential in depolarized ventricular cells treated with Ba^{2+} is effectively decreased by benidipine (Kodama *et al.*, 1987). A receptor binding study indicates that benidipine interacts with the binding site of 1,4-dihydropyridine (DHP) Ca antagonists as determined by [^3H]-nitrendipine (Ishii *et al.*, 1988a). It is, therefore, very likely that benidipine binds to the same site and blocks voltage-dependent Ca channels in the same manner as other DHP Ca antagonists.

The long-lasting antihypertensive effect of benidipine cannot be explained by its rate of metabolism *in vivo* (Kobayashi *et al.*, 1988). Moreover, it has been found that the removal of these inhibitory effects of benidipine after the withdrawal of the drug is extremely slow even *in vitro* (Terada *et al.*, 1987; Karasawa & Kubo 1988; Gotoh *et al.*, 1989). These observations suggest that the long-lasting antihypertensive action of benidipine is, at least in part, attributable to very slow dissociation of the drug molecules from target cells. It is, however, still open to question whether the slow recovery from benidipine-induced inhibition can be simply explained by a slow dissociation of the molecule from DHP binding sites in voltage-dependent Ca channel. The present study was undertaken to elucidate the mechanisms of the slow recovery of I_{Ca} from the block by use of single cardiac cells isolated from ventricle of the guinea-pig. This preparation was chosen because the I_{Ca} is large under physiological conditions. In arterial smooth muscle cells, substantial I_{Ca} can be recorded only in solutions containing a high concentration of Ca or Ba

(Bean *et al.*, 1986; Terada *et al.*, 1987; Yatani *et al.*, 1987), which competes with (Lee & Tsien, 1983) and may change the kinetics of Ca-antagonists including DHP-derivatives.

Methods

Cell isolation

The dissociation procedure was very similar to that described by Giles & Imaizumi (1988). In brief, hearts were removed from male guinea-pig weighing 200 – 300 g and mounted on a modified Langendorff perfusion system for retrograde perfusion of the coronary circulation. The following procedure was used: (1) perfusion with the normal Krebs solution for about 10 min; (2) perfusion for approx. 15 min with Ca-free Krebs solution; (3) perfusion for 10 – 15 min with Ca-free Krebs solution containing about 0.04% collagenase (Yakult, Tokyo). Following this, the entire ventricular wall was dissected into pieces and placed in a small vessel. These pieces were incubated in a HEPES-buffered solution which contained 0.01% collagenase. After gentle stirring, a large number of single ventricular cells were released. The cell suspension was then diluted about 30 times in the HEPES-buffered solution and kept at room temperature. The composition of solutions used in this procedure is as follows (mM): Krebs solution: Na^+ 137.0 , K^+ 5.9 , Ca^{2+} 2.2 , Mg^{2+} 1.2 , Cl^- 123.5 , HCO_3^- 25 , H_2PO_4^- 1.2 , glucose 14.0 ; HEPES-buffered solution: Na^+ 137.7 , K^+ 5.7 , Ca^{2+} 0.05 , Mg^{2+} 0.6 , Cl^- 143.7 , HPO_4^{2-} 0.35 , H_2PO_4^- 0.3 , glucose 20.0 , HEPES 20.0 . The pH of the HEPES-buffered solution was adjusted at 7.4 with NaOH. Krebs solution was equilibrated with a mixture of 95% O_2 and 5% CO_2 resulting in pH 7.4 at 37°C . HEPES-buffered solution was gassed with 100% O_2 .

Recording techniques

An aliquot of this cell suspension was placed in a recording chamber having a volume of 0.5 ml and mounted on the stage

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of an inverted microscope (TMD, Nikon). Cells were continuously perfused with solutions at 5 ml min^{-1} . Experiments were carried out in a bathing solution of the following composition (mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.2, Mg^{2+} 1.2, Cl^- 149.7, glucose 14 and HEPES 10. The pH was adjusted to 7.2 with NaOH. Measurement of ionic currents was performed by the tight-seal whole-cell clamp technique (Hamill *et al.*, 1981). Pipette resistances ranged from 2 to 5 M Ω when filled with a pipette solution containing (mM): K^+ 150, Na^+ 8, Mg^{2+} 1.0, aspartate 120, Cl^- 32.0, adenosine triphosphate (ATP) 4, ethyleneglycol-bis-(B'-amino-ethyl ester) N,N,N',N'-tetraacetic acid (EGTA) 0.3 and HEPES 5. The pH of the pipette solution was adjusted to 7.2 with KOH. The series resistance was partly compensated electrically. The amplifier used here was the same circuit described previously (Clark & Giles, 1987) or List EPC-7. All procedures for approaching the cell, making G Ω seal and rupturing the cell membrane were done under $\times 800$ magnification (TMD, Nikon). Electrophysiological measurements were carried out at $30 \pm 2^\circ\text{C}$. The resting membrane potential of single ventricular cells used in this study was $-74.1 \pm 0.5 \text{ mV}$ ($n = 15$). The zero-current level under voltage clamp mode was determined from the level when clamped at the resting membrane potential in each cell.

Data storage and analysis

Data storage and analysis were performed as previously described by Imaizumi *et al.* (1989). In brief, electrical events were monitored on a storage oscilloscope (VC-6041, Hitachi, Tokyo, Japan) and stored on video-tape after being digitized by a PCM-recording system (PCM 501ES, SONY, TOKYO, JAPAN; modified to obtain a frequency-response from d.c. to 20 kHz). The data on the tape were replayed later and loaded into a computer (IBM-AT) through A-D converter (Data Translation; DT2801A) for analysis. Data-acquisition (AQ, Robinson & Giles, 1986) and -analysis software (DAS) for IBM-AT were supplied by Dr Wayne Giles (Univ. Calgary, Canada). Selected records were printed out with a laser printer (HP Laser Jet Series II).

Drugs

Benidipine hydrochloride (1,4 dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid methyl-1-(phenylmethyl)-3-piperidinyl ester hydrochloride) and nifedipine were synthesized and supplied by Kyowa Hakko (Tokyo, Japan). CdCl_2 (Wako Pure Chemical Industries, Ltd. Osaka, Japan), tetrodotoxin (Sigma) and EGTA (Dojin, Kumamoto, Japan) were also used.

Statistics

The values are expressed as mean $\pm$ s.e.mean in the text and figures. Statistical significance was determined by Student's *t* test and indicated by * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

Results

Blocking action of benidipine and nifedipine on I_{Ca}

Figure 1a (open circle) shows a typical recording of inward current elicited by depolarization from a holding potential of -40 mV to $+10 \text{ mV}$ in an isolated ventricular cell. The maximum of peak inward current was obtained near $+10 \text{ mV}$ as illustrated in current-voltage relationship (I-V curve, Figure 1b; open circles). The averaged peak amplitude of the inward current at $+10 \text{ mV}$ was $527.2 \pm 43.8 \text{ pA}$ ($n = 20$) when a depolarizing pulse was applied at 0.2 Hz . A significant contamination by Na current of this inward current can be ruled out, since an application of $10 \mu\text{M}$ tetrodotoxin did not apparently affect the amplitude of the inward current under these conditions (not shown). The I_{Ca} indicated by closed triangles in Figure 1a was recorded about 10 min after the application

of 10 nM benidipine. Benidipine decreased I_{Ca} at any voltages between -10 and $+40 \text{ mV}$ as shown in the I-V curve (Figure 1b, closed triangles). Since the total membrane current includes a time-independent inward rectifier K current and a leakage current, the amplitude of the I_{Ca} cannot be accurately measured with respect to the zero current level. Therefore, the amplitude of I_{Ca} was measured as the deviation between the peak and the zero I_{Ca} level which was detected by complete block of I_{Ca} or approximated by the level at the end of a 150 ms pulse at $+10 \text{ mV}$ in the presence of a Ca antagonist. When the I_{Ca} was completely blocked, the current level was almost steady during the depolarizing pulse of 150 ms in the presence of a Ca antagonist (cf. Figure 1a closed triangle). Since the onset of the time- and voltage-dependent K current is slow in this preparation (Cohen *et al.*, 1986), the error including this K current is negligible when the amplitude of I_{Ca} is determined at the peak. After treatment with 10 nM benidipine for approximately 15 min, the peak I_{Ca} at $+10 \text{ mV}$ was decreased to $25.3 \pm 7.4\%$ ($n = 9$) of that before the treatment.

Figure 1c illustrates the time course of the onset of the effect of 10 nM benidipine. While I_{Ca} was elicited at 0.2 Hz , all data were not plotted to make the figure simple. There appeared to be a delay of decrease in I_{Ca} after the application of 10 nM benidipine. The rate of decrease in I_{Ca} became slow after about 5 min from the start of the treatment with benidipine and was

Figure 1 Effects of 10 nM benidipine on the inward current (I_{Ca}) elicited by depolarization from -40 to $+10 \text{ mV}$ at 0.2 Hz . (a) Upper panel indicates the pulse applied. Three traces ($\circ$: control; $\blacktriangle$: in the presence of 10 nM benidipine; $\triangle$: 30 min after washout of benidipine) were superimposed in the lower panel. (b) The peak amplitude of I_{Ca} recorded at various voltages in the same way as shown in (a) was plotted as current-voltage relationship. Symbols are the same as in (a). (c) The onset time course of I_{Ca} inhibition by 10 nM benidipine. Closed symbols indicate the peak amplitude of I_{Ca} in the presence of 10 nM benidipine. Although I_{Ca} s were elicited at 0.2 Hz , not all the data were plotted to simplify the figure.

close to a steady level after 10 min ($n = 4$). The onset of blockade by 100 nM nifedipine was much faster and the effect reached a steady level within 3 or 4 min (not shown, $n = 5$). At lower concentrations (1 and 3 nM), benidipine-induced block appeared more slowly. Since it was difficult to separate clearly the effect of benidipine from 'run-down' of I_{Ca} after the initial marked development of the blockade, the effect of benidipine was determined approximately 15 min after the start of the application.

The current trace indicated by the open triangle in Figure 1a was recorded approximately 30 min after the start of withdrawal of 10 nM benidipine. I_{Ca} did not recover but further decreased. Recovery of I_{Ca} from benidipine-induced block was not observed at least within 30 min ($n = 8$) under these conditions. It confirms the previous observation that Ba^{2+} current hardly recovered after withdrawal of benidipine in smooth muscle cells from rabbit mesenteric artery (Terada *et al.*, 1987). It might be postulated that significant 'run-down' of I_{Ca} occurred and masked the recovery from blockade during the washing. This possibility cannot be ruled out completely but is rather unlikely, because the I_{Ca} was transiently increased by hyperpolarization to near the control level even under these conditions (see Figures 4, 5 and 6, and corresponding parts in the text).

Figure 2 (triangles) illustrates the dose-response relationship between benidipine and the I_{Ca} at +10 mV. The approximate IC_{50} was 2.7 nM. Circles in Figure 2 denote the relationship for nifedipine determined under the same conditions. The IC_{50} of nifedipine was 63.1 nM.

Effects of hyperpolarization on the blockade by benidipine

It has been reported that the I_{Ca} blockade by DHP Ca-antagonists is strongly dependent upon holding potential (Bean, 1984; Sanguinetti & Kass, 1984). When a cell is kept at a very negative potential, the blockade is small since the affinity of these drugs for Ca channels in the resting state is rather small. The blockade by DHP Ca antagonists is also removed by hyperpolarization. Experiments were carried out to examine whether benidipine has the same character as

Figure 2 Dose-response curves of nifedipine (●) and benidipine (▲) for the inhibition of I_{Ca} in single ventricular cells. The I_{Ca} was elicited by depolarization from -40 to $+10$ mV in the same manner in Figure 1. The I_{Ca} obtained before the application of the drugs was taken as 1.0. The effects of the drugs were determined after an application for 10–15 min. The symbols and vertical bars indicate the mean and s.e.mean, respectively. The number of observations was 4–7 for each point.

Figure 3 (a) The protocol to examine the effects of hyperpolarization on the recovery of I_{Ca} . The I_{Ca} s were elicited by depolarization from -40 to $+10$ mV at 0.2 Hz until the amplitude reached a steady level ($n > 20$). Thereafter, the cell was hyperpolarized to -75 mV and kept quiescent for 5 min without any stimulation. After the holding potential was returned to -40 mV for 10 s, the same depolarizing pulses were again applied at 0.2 Hz for 3 min ($m = 34$). (b) The relative amplitude of I_{Ca} was plotted against the time measured. Circles, triangles and squares indicate the means of the relative I_{Ca} in the absence ($n = 5$) and presence of 100 nM nifedipine ($n = 5$) and 10 nM benidipine ($n = 5$), respectively. The I_{Ca} amplitude at time 0 was taken as 1.0 in the absence or presence of nifedipine or benidipine, respectively. To make the figure simple, not all the data were plotted. (c) Typical recordings obtained in the absence (i), or presence of 100 nM nifedipine (ii) or 10 nM benidipine (iii), respectively. Five traces obtained at the times indicated by arrows were superimposed.

nifedipine in this respect. Figure 3 illustrates the protocol (a) and shows the relative amplitude of I_{Ca} (b) in the absence (open circles) and presence of 100 nM nifedipine (closed triangles) or of 10 nM benidipine (closed squares). After the effect of nifedipine or benidipine on I_{Ca} elicited by depolarization from a holding potential of -40 to $+10$ mV at 0.2 Hz reached a steady level (time zero), the holding potential was changed to -75 mV for 5 min. Then, depolarizing pulses were again applied at 0.2 Hz after the holding potential had been returned to and kept at -40 mV for 10 s. Although the potential of -75 mV is close to the resting membrane potential, the change in holding potential from -40 to -75 mV as well as to -100 mV (see Figure 4) is called hyperpolarization in the present study for convenience. Some of the Ca channels are inactivated at -40 mV as the steady-state inactivation (Isenberg & Klöckner, 1982) and also by depolarizing pulses. In preliminary experiments, the recovery time course of I_{Ca} from inactivation was examined by changing the duration of the hyperpolarization (-75 mV). In these experiments, the application of depolarizing pulses (from -40 to $+10$ mV; 0.2 Hz) was started 30 ms after the change of holding potential from -75 to -40 mV. It was found that I_{Ca} amplitude

Figure 4 The effects of hyperpolarization on the recovery of I_{Ca} after the withdrawal of 100 nM nifedipine (a) or 10 nM benidipine (b). The protocol was the same as shown in Figure 3, except that the Ca antagonists were withdrawn when the hyperpolarization was applied. Moreover, the cells were hyperpolarized to -75 (○) or -100 mV (■) or not hyperpolarized (-40 mV, ▲) for 5 min. A typical result is shown for each procedure. (ii) The traces recorded at time 0. (iii) Five traces recorded at the times indicated by arrows were superimposed.

elicited by the first depolarizing pulse after the hyperpolarization was almost steady when the duration of the hyperpolarization was longer than 20 s in the absence or presence of 10 nM benidipine or 100 nM nifedipine ($n > 3$ for each). Therefore, hyperpolarization for 5 min was apparently enough to reach the steady level of recovery even in the presence of drugs. In the protocol shown in Figure 3a, however, the voltage-dependent inactivation of Ca channels again developed during 10 s at -40 mV after the hyperpolarization. Therefore, the I_{Ca} elicited by the first pulse after the hyperpolarization was increased to only 111% of that before the hyperpolarization in the absence of drugs (see Figure 3b open circles). By these means, the development of re-block of I_{Ca} by DHPs was distinguished from the voltage-dependent inactivation which mostly finished during the period of 10 s at -40 mV. Figure 3c shows typical recordings obtained at the arrows in the absence (ci), or presence of 100 nM nifedipine (cii) or 10 nM benidipine (ciii) after the hyperpolarization. The values were normalized taking the amplitude before the hyperpolarization as 1.0 in the presence or absence of 100 nM nifedipine or 10 nM benidipine, respectively. The absolute values of I_{Ca} at time 0 in the absence and presence of 100 nM nifedipine and 10 nM benidipine were 488.3 ± 78.9 ($n = 5$), 201.3 ± 41.9 ($n = 5$) and 142.1 ± 30.0 pA ($n = 5$), respectively. The I_{Ca} elicited by the first pulse after the hyperpolarization was 535.5 ± 75.4 ($n = 5$), 383.0 ± 77.4 ($n = 5$) and 413.2 ± 70.4 pA ($n = 5$) in the absence or presence of 100 nM nifedipine and 10 nM benidipine, respectively. It appears clear that the hyperpolarization significantly reduced nifedipine and benidipine-induced block. If hyperpolarization caused the recovery of unblocked and inactivated channels so that they became available, the rate of recovery of I_{Ca} at the first pulse after the hyperpolarization should be the same in the absence or the presence of drugs. This was apparently not the case since the rate of recovery was significantly greater in cells treated with 100 nM nifedipine or 10 nM benidipine. The removal of the block by hyperpolarization was more apparent in cells treated with 10 nM benidipine than 100 nM nifedipine. The relative amplitude of the first I_{Ca} after hyperpolarization was 1.11 ± 0.08 ($n = 5$), 1.88 ± 0.15 ($n = 5$, $P < 0.01$ vs. control), 3.15 ± 0.27 ($n = 5$, $P < 0.001$ vs. control; $P < 0.01$

vs. nifedipine) in the absence (control) or presence of 100 nM nifedipine or 10 nM benidipine, respectively (see Figure 5). Figure 3b (triangles and squares) also illustrates the time course of the re-block of I_{Ca} by DHP Ca antagonists.

Sustained block by benidipine after withdrawal

If hyperpolarization accelerates the dissociation of DHP Ca antagonist from the binding site, it should enhance recovery from blockade after withdrawal of the drug. This possibility was examined by use of the same protocol as shown in Figure 3, except that the hyperpolarization was started at the same

Figure 5 The summarized data obtained in experiments shown in Figures 3 and 4. Open and closed columns indicate the relative amplitude of I_{Ca} obtained at 5 and 8 min. The I_{Ca} recorded at time 0 in absence or presence of Ca blockers was taken as 1.0 in each preparation. (a) In the absence of Ca antagonist. (b and c) Nifedipine (100 nM) or 10 nM benidipine was present throughout the experiments, respectively. (d and e) Nifedipine (100 nM) or 10 nM benidipine was withdrawn when the cells were hyperpolarized to -75 mV. The statistical significance between columns is shown in the upper part of the figure. The number of observations was five for each.

time as withdrawal of DHPs (Figure 4). The removal of the effect of 100 nM nifedipine after withdrawal was clearly enhanced by hyperpolarization. When the cell was not hyperpolarized (-40 mV, triangles in Figure 4a(i)), the recovery of I_{Ca} was not apparent after 8 min. In cells hyperpolarized to -75 mV (circles) or -100 mV (squares), I_{Ca} clearly recovered after 8 min in Figure 4a(i). Figure 4a(ii) and (iii) show the current traces recorded at time 0 and the time indicated by arrows in Figure 4a(i), respectively. In contrast with nifedipine, the removal of benidipine-induced block after withdrawal was not accelerated by hyperpolarization (Figure 4b). Although the first I_{Ca} after the hyperpolarization recovered significantly (square and circle at 5 min), the re-block of I_{Ca} developed to the level similar to those at time 0, regardless of the potential of hyperpolarization. Figure 4b(ii) and (iii) shows the recordings at time 0 and the time indicated by arrows in Figure 4b(i), respectively. It is notable that the rate of re-block was time-dependent and quite close to that in the presence of benidipine (Figure 3b and 4b(i)). Figure 5 illustrates the summarized data obtained after the hyperpolarization to -75 mV. The amplitude of I_{Ca} at time 0 in Figure 3b, 4a(i) and 4b(i) was taken as 1.0 in the same manner, respectively. Open and closed columns denote the relative I_{Ca} s at 5 (the first I_{Ca} after the hyperpolarization) and 8 min, respectively. In Figure 5, a, b, c, d and e denote the relative I_{Ca} in the control, in the presence of 100 nM nifedipine and 10 nM benidipine, and after the withdrawal of nifedipine and benidipine, respectively. It is clear that the blockade induced by 100 nM nifedipine was significantly removed by hyperpolarization after withdrawal (closed columns in (b) and (d)), whereas that by 10 nM benidipine was not at all (closed columns in (c) and (e)).

It can be postulated that the duration of hyperpolarization (5 min) is too short for dissociation of benidipine. Recovery of I_{Ca} was, however, not observed clearly even when the hyperpolarization was prolonged up to 15 min (not shown) or applied twice (Figure 6) after the withdrawal of benidipine. There were considerable difficulties in recording constant I_{Ca} for over 40 min because of the run-down of I_{Ca} (Belles *et al.*, 1988), the breakdown of the G Ω seal, an increase in series resistance, etc. Therefore, it is quite rare to obtain continuous good recordings of I_{Ca} including before and after the application of the drugs and after withdrawal. Figure 6 shows typical time courses of changes in I_{Ca} amplitude. The I_{Ca} before the application of drugs was taken as 1.0 (426 pA in a and 604 pA in b). Cells were hyperpolarized twice after the withdrawal of drugs. In cells treated with nifedipine (Figure 6a), the I_{Ca} recovered to approximately 80% (indicated by an arrow) of the control after the withdrawal. In contrast, the block by 10 nM benidipine was transiently removed by hyperpolarization but re-developed within 5 min (indicated by the arrows in b) even after the second hyperpolarization. Similar results were obtained in three cells each for nifedipine and benidipine.

Effects of benidipine applied intracellularly

It is possible that benidipine cannot be removed by washing because it binds to Ca channels from the inside of the cell membrane. In order to examine this possibility, $1 \mu\text{M}$ benidipine was added to a pipette solution and applied intracellularly by simple diffusion. After rupturing the cell membrane at the holding potential of -75 mV to make a whole cell clamp configuration, the potential was immediately changed to -40 mV. The first depolarizing pulse was applied about 20 s after rupturing the cell membrane. Figure 7 illustrates the time course of peak I_{Ca} amplitude (Figure 7a; 0.2 Hz) after the rupture and current traces (Figure 7b) recorded at the beginning and after 3.5 min as indicated by arrows (Figure 7a(i) and (ii)). The amplitude of I_{Ca} increased and reached a maximum after 3–5 min. Thereafter, I_{Ca} decreased gradually at a rate similar to or slightly faster than the usual rate of I_{Ca} run-down (approximately 5–15% per 10 min). In three cells examined,

Figure 6 Effects of hyperpolarization to -75 mV in the presence and after the withdrawal of 100 nM nifedipine (a) and 10 nM benidipine (b). The protocol was the same as shown in Figure 3a. The I_{Ca} amplitude before the application of Ca antagonists was taken as 1.0 (426 pA in a and 604 pA in b). For significance of arrows, see the text.

this small enhancement of I_{Ca} was observed and the averaged maximum enhancement of I_{Ca} was $9.4 \pm 4.0\%$ ($n = 3$). When a pipette solution did not include benidipine, the peak amplitude of I_{Ca} declined to $76.2 \pm 2.0\%$ ($n = 6$; $P < 0.001$ vs. $109.4 \pm 4.0\%$) during the first 5 min. This decay includes the development of I_{Ca} inactivation after the change of holding potential to -40 mV and run-down. Thereafter, the rate of run-down was mostly constant (approximately 5–15% per 10 min). Similar intracellular application of Bay K 8644 ($1 \mu\text{M}$

Figure 7 Effects of benidipine applied to the pipette solution ($1 \mu\text{M}$). (a) The time course of changes in the relative amplitude of I_{Ca} . About 20 s before time 0, the cell membrane was ruptured and then the whole cell clamp was performed. (b) The current traces obtained at the times indicated by (i) and (ii) in (a).

in a pipette solution, $n = 1$) or cyclic AMP (300 μM in a pipette solution, $n = 2$) markedly increased the I_{Ca} by 50–100%. The potentiation by Bay K 8644 and cyclic AMP had a faster and slower onset-time course respectively than that by benidipine.

Discussion

It has been clearly shown that benidipine is more potent than nifedipine as a Ca antagonist. The IC_{50} s of nifedipine and benidipine obtained under the conditions used in the present study (holding potential: -40 mV , depolarization by 50 mV, $[\text{Ca}]_0 = 2.2\text{ mM}$, 0.2 Hz) were 63.1 and 2.7 nM, respectively. It has been reported that the blockade by DHP Ca-antagonists depends strongly upon the holding potential (Bean, 1984; Sanguinetti & Kass, 1984). The IC_{50} s may be larger than these values if more negative holding potentials are used (see Terada *et al.*, 1987). The potency ratio between nifedipine and benidipine (23.3 when determined from IC_{50} s) might be slightly smaller when the holding potential is more negative, since the removal of blockade during long hyperpolarization appeared to be greater when treated with benidipine than nifedipine (see Figure 5; open columns in (b) and (c)). The IC_{50} of nifedipine determined under similar conditions (holding potential: -40 mV , $[\text{Ca}]_0 = 10\text{ mM}$) in the same preparation is approximately 150 nM (Lee & Tsien, 1983). Since higher $[\text{Ca}]_0$ decreases the nifedipine-induced blockade (Lee & Tsien, 1983), the difference in IC_{50} s of nifedipine in these two studies may be reasonable.

The two major and quite important findings in the present study are as follows. (1) The blockade of I_{Ca} by benidipine at low concentrations was transiently removed by hyperpolarization as was that by nifedipine. (2) The removal of blockade by nifedipine after withdrawal was markedly enhanced by hyperpolarization. In contrast, benidipine-induced blockade was not removed or quite slowly removed even when prolonged hyperpolarization was applied after withdrawal.

It has been suggested that DHP Ca-antagonists have the highest affinity for Ca channels in an inactivated state and that hyperpolarization enhances the dissociation rate of the drugs (Bean, 1984; Sanguinetti & Kass, 1984), whereas the binding to the channels in the resting state also occurs (Uehara & Hume, 1985). This may explain the marked enhancement of the removal of nifedipine-induced block by hyperpolarization after withdrawal of the drug. It can be strongly suggested that nifedipine molecules which dissociate from the DHP-binding site during hyperpolarization are easily washed away from the Ca channel. Therefore, the simplest explanation for the sustained effect of benidipine after withdrawal may be as follows. The hyperpolarization enhances the dissociation of benidipine from DHP-binding site as well as nifedipine and increases the number of unblocked channels at the resting state. This results in the marked recovery of I_{Ca} elicited by the first depolarizing pulse after long hyperpolarization (Figures 3 and 4). Even after the benidipine molecule dissociates from the DHP-binding site during hyperpolarization, however, it may still keep interaction with the channel somehow and not be washed away easily. When the holding potential is returned to -40 mV , the benidipine molecule may preferentially bind again to the DHP-binding site for blockade of the Ca channel in the inactivated state (Figures 4 and 5). It is likely that benidipine has an affinity for additional site(s) in the Ca channel or surrounding lipid layer because of its lipophilic nature (Suzuki *et al.*, 1988). Such a possibility has been suggested for other lipophilic DHP compounds (Kokubun & Reuter, 1984). Valdivia & Coronado (1988) have suggested that the DHP receptor is buried in the lipid bilayer adjacent to the external end of the Ca channel.

An alternative explanation for the sustained interaction between benidipine and the Ca channel may be as follows. The hyperpolarization for several minutes may have little

effect on the dissociation of benidipine from the DHP binding site because the dissociation rate is so low in comparison with nifedipine. It has been reported that the inhibitory effects of benidipine at low concentrations ($<0.3\text{ nM}$) in vascular strips are partly removed by washing for over an hour (Gotoh *et al.*, 1989). There is a possibility, therefore, that the difference between benidipine and nifedipine which is responsible for the different recovery is only the rates of dissociation from the DHP-binding site which directly correlates with the blocking action. This hypothesis, however, requires a unique assumption that the Ca channel bound by benidipine can recover from inactivation during hyperpolarization and can be open by the next depolarization. The following opening and inactivation of the channel might lead to the blockade again.

In connection with the foregoing argument, an interesting hypothesis can be postulated that the transient but marked recovery of I_{Ca} after hyperpolarization in the presence of benidipine is due to a transient potentiation of I_{Ca} by the drug rather than removal of the block. It has been pointed out that benidipine enhances I_{Ba} slightly in vascular smooth muscle cells under certain conditions (Terada *et al.*, 1987). An apparent potentiation was not observed in the present study because the holding potential used was mostly less negative (-40 mV) than that required to show the potentiation (Terada *et al.*, 1987; for other DHP compounds, Brown *et al.*, 1984; 1986; Kokubun, 1984). This mechanism, however, appears to be unlikely because of the following. First, the potentiation of I_{Ca} by benidipine appears to be small (Terada *et al.*, 1987). Second, the potentiation may not occur after the cell is clamped at -40 mV for 10 s (see text), if the mechanism of the potentiation by benidipine is similar to that by nitrendipine (Brown *et al.*, 1986). Third, the transient recovery of I_{Ca} was also observed in cells treated with nifedipine. Further experiments are required to clarify the possibility.

Two types of Ca channels have been identified in cardiac cells (Bean, 1985; Nilius *et al.*, 1985) as well as multitypes of Ca channels in other excitable cells (Hagiwara *et al.*, 1975; Nowicky *et al.*, 1985). It has been suggested that T type channels have low sensitivity to DHP Ca antagonists (Nowicky *et al.*, 1985). It might therefore be possible that the partial recovery of I_{Ca} by hyperpolarization in the presence of the DHP Ca antagonist is not due to the removal of block of L type Ca channels but the recovery of T type channels from voltage-dependent inactivation. However, during the period of 10 s at -40 mV after the hyperpolarization, most T type channels must be inactivated (Bean, 1985; Fox *et al.*, 1987). Therefore, the contribution of I_{Ca} through T type Ca channels to the recovery of I_{Ca} after the hyperpolarization can be ruled out.

A pharmacodynamic study has shown that the half decay time of plasma-concentration of benidipine after oral or intravenous administration is not long enough to explain its long lasting antihypertensive effect (Kobayashi *et al.*, 1988). The dissociation of both $[\text{^3H}]$ -benidipine from its binding site and benidipine from the $[\text{^3H}]$ -nitrendipine binding site is quite slow (Karasawa & Kubo, 1988; Ishii *et al.*, 1988b). Moreover, an autoradiographic study of the distribution of $[\text{^3H}]$ -benidipine has shown that the amount of ^3H remaining in the tissues after the plasma concentration of benidipine decreases is not particularly large. In the present study, a sustained interaction between benidipine and Ca channel after withdrawal of the drug was shown electrophysiologically. Therefore, it is fairly clear that the sustained interaction is essentially responsible for the long-lasting antihypertensive effect of benidipine. However, the exact site in the Ca channel or its surroundings for the sustained interaction with benidipine was not determined in the present study. A question whether the site is specific for benidipine or not, correlates directly with another—whether the difference between nifedipine and benidipine as to the recovery from their blockade is qualitative or quantitative. In the preceding paragraphs, two possibilities were put forward. First, the site might be most simply the DHP-binding site which is responsible for the blockade. Benidipine may have a high affinity and an

extremely low rate of dissociation to the site. Second, the site may be an additional binding site in Ca channel protein or more probably the surrounding lipid layer. In addition to these, another possibility might be suggested. The apparent pK_a of benidipine has been calculated as 7.34 (Suzuki *et al.*, 1988) and is much larger than that of nifedipine (pK_a ~1; Uehara & Hume, 1985). It indicates that half of the dissolved benidipine molecules are ionized in a solution at pH 7.4. The ionized benidipine might interact with the negative charge in the Ca channel protein or surrounding protein on the cell membrane. It is, however, rather unlikely that an ionized molecule has a sustained interaction with the negative charge on the membrane when it acts from outside the cell membrane. The possibility should be examined at different pH values which change the ratio of neutral and ionized molecules.

The consideration mentioned above reminds us of the mechanism of Ca channel block by verapamil and D-600 which have pK_a of 8.7 (Sanguinetti & Kass, 1984). These Ca antagonists interact with their binding site in the Ca channel from inside the cell membrane when the channel opens since the molecules are mostly ionized at pH 7.2 (Hescheler *et al.*, 1982). Intracellularly applied benidipine, however, did not apparently block I_{Ca}. Therefore, the possibility that benidipine

interacts with the binding site of DHP Ca-antagonist from inside the cell membrane is completely ruled out. It is, however, likely that benidipine activates the Ca channel when applied internally in the same way as Bay K 8644 does. Another possible mechanism of the potentiation by benidipine might be an inhibition of cyclic AMP phosphodiesterase by benidipine. This mechanism has been suggested for some other DHP Ca antagonists (Iijima *et al.*, 1984), while the activity of benidipine as an inhibitor has not been examined.

In conclusion, the blocking effect of benidipine on voltage-dependent Ca channels was much stronger than that of nifedipine. It is suggested that hyperpolarization enhanced the dissociation of benidipine, as well as nifedipine, from the DHP-binding site. The removal of blockade by nifedipine after its withdrawal from the solution was significantly enhanced by hyperpolarization but that by benidipine was not. There may be an additional interaction between benidipine and the Ca channel or surrounding lipid which keeps the molecule near the Ca channel in a voltage-independent manner even after withdrawal of benidipine from the solution. Alternative explanations of the mechanisms of sustained interaction could not be ruled out completely in the present study.

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