Voltage- and Use-Dependent Block of the Inward Calcium Current by MCI-176, a New Non-Dihydropyridine Calcium Antagonist, in Canine Ventricular Muscles and Single Ventricular Cells of the Guinea-Pig

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Abstract—Effects of MCI-176, 2-(2,5-dimethoxyphenylmethyl)-3-(2-dimethylaminoethyl)-6-isopropoxy-4(3H)-quinazolinone hydrochloride, on action potentials of canine ventricular muscles and on membrane currents of single ventricular cells of the guinea-pig heart were studied with the microelectrode and the patch-clamp ("whole-cell recording") methods. In canine ventricular trabeculae, MCI-176 (10^{-5} – 10^{-4} M) decreased the plateau potential, the action potential duration at 30%-repolarization and the maximum rate of rise of the action potential; and it also decreased the amplitude and the duration of the slow response action potential in a concentration-dependent manner. Those effects were much more apparent at higher stimulus frequency. Under voltage clamp condition of single ventricular cells of the guinea-pig heart, MCI-176 (3×10^{-5} M) decreased the inward calcium current (I_{Ca}) by 25–30% when the membrane potential was held at the resting membrane potential, and the drug abolished it when the membrane potential was held at -30 mV. MCI-176 added at rest decreased I_{Ca} ("initial block") and reduced it further with repetitive depolarizations in a beat-to-beat fashion. MCI-176 facilitated the reduction of I_{Ca} by increasing the clamp pulse frequency. These results indicate that MCI-176 decreases I_{Ca} of mammalian ventricular muscles in a voltage- and use-dependent manner.

Calcium antagonists have recently been established as an important class of drugs with a wide spectrum of therapeutic effects on cardiovascular diseases. They are structurally heterogeneous and can be classified as papaverine, dihydropyridine and benzothiazepine derivatives. A previous study (1) has demonstrated that KB-944, a benzothiazol benzylphosphonate derivative (2), has a selective calcium channel blocking action. It seems likely that these compounds are not uniformly effective against all voltage-sensitive calcium channels (3). Dihydropyridine calcium antagonists are less cardio-depressant or more vasoselective than non-dihydropyridine calcium antagonists. This selectivity may arise from different affinities for calcium channels in different tissues, but

precise mechanisms are still unclear. It is, therefore, very important to find a new calcium antagonist which is structurally unrelated to the existent compounds for studying the physiological and pharmacological natures of calcium channels.

MCI-176, 2-(2,5-dimethoxyphenyl-methyl)-3-(2-dimethylaminoethyl)-6-isopropoxy-4(3H)-quinazolinone hydrochloride, is a newly developed calcium channel blocker which is one of the quinazolinone derivatives (4). The cardiovascular profile of MCI-176 has been studied in isolated canine heart preparations, and it has been elucidated that MCI-176 is a non-vasoselective calcium antagonist like other non-dihydropyridine calcium antagonists (5). However, none of reports have demonstrated the selective

blockade by MCI-176 of the inward calcium current (I_{Ca}). Therefore, the present experiments were carried out to reveal the effects of MCI-176 on the membrane potential of canine ventricular trabeculae and on membrane currents of isolated ventricular cells of the guinea-pig with the conventional micro-electrode technique and the patch clamp technique in the whole cell recording configuration, respectively.

Materials and Methods

Canine ventricular muscles: Mongrel dogs (6–16 kg) of either sex were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). The hearts were rapidly excised and placed in cold (5–10°C) Tyrode's solution equilibrated with 95% O₂ and 5% CO₂. The right ventricular trabeculae, about 1 mm in diameter and 3–5 mm in length, were separated and pinned to the bottom of a tissue chamber (3 ml volume). The preparation was superfused at a rate of 6 ml/min with Tyrode's solution containing (in mM): K⁺, 2.7; Na⁺, 149.3; Ca²⁺, 1.8; Mg²⁺, 1.0; Cl⁻, 145.3; HCO₃⁻, 11.9; H₂PO₄⁻, 0.4; and dextrose, 5.5, equilibrated with 95% O₂ and 5% CO₂. The pH of the bathing solution ranged from 7.2 to 7.4. Tissue bath temperature was maintained at 37±0.5°C. The preparation was allowed to equilibrate for at least 1 hr before membrane potentials were recorded. Slow response action potentials were induced by superfusing the tissue with 10⁻⁶ M isoproterenol-containing 27 mM K⁺ Tyrode's solution which was prepared by equimolar substitution of KCl for NaCl; the sum of K⁺ and Na⁺ concentration was constant at 152.0 mM. Membrane potentials were recorded with glass microelectrodes filled with 3 M KCl, having resistances of 10–15 meg-ohm.

Single ventricular cells of the guinea-pig: Single ventricular cells of the guinea-pig heart were obtained by an enzymatic dissociation method (6). The preparation of single cells and the technique of whole-cell recording are essentially the same as in a previous report (7). Briefly, the heart was perfused through the coronary arteries with Ca-free Tyrode's solution containing 0.04% (w/v) collagenase (Sigma, type 1) for 40–60 min at 36°C. The heart was then stored in the storage solution (8) at 4°C for at least 1 hr. A

small piece of the ventricular tissue was dissected and was gently agitated in the recording chamber (0.5 ml in volume) filled with normal Tyrode's solution. The recording chamber was perfused at a rate of 2–3 ml/min with normal Tyrode's solution after the cell had settled on the floor of the chamber. Experiments were carried out at 36–37°C on rod-shaped quiescent single cells which have the clear striation of sarcomeres. Normal Tyrode's solution contained (in mM): NaCl, 136.9; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 0.53; NaH₂PO₄, 0.33; and glucose, 5.5; and the pH was adjusted to 7.4 with 5 mM HEPES-NaOH buffer. The storage solution contained (in mM): taurine, 10; oxalic acid, 10; glutamic acid, 70; KCl, 25; KH₂PO₄, 10, and HEPES-KOH, 10 (pH=7.4). The standard pipette solution contained (in mM): K-aspartate, 110; KCl, 20; MgCl₂, 2; ATP (dipotassium salt), 4; creatine phosphate (disodium salt), 6; EGTA, 0.05; and HEPES-KOH, 5 (pH=7.0). The Cs⁺ pipette solution was prepared simply by substituting Cs⁺ for K⁺ in the standard pipette solution except 2Na-ATP.

Results

Effects on normal action potentials of canine ventricular muscles: Effects of MCI-176 on normal action potentials were studied in canine ventricular trabeculae electrically stimulated at a rate of 0.5 and 1.0 Hz. At a low stimulus frequency of 0.5 Hz, slight decreases in the maximum rate of rise and the action potential duration measured at the 30%-repolarization level were seen with 3×10⁻⁵ M of MCI-176. Such effects were slightly greater at 10⁻⁴ M. With increasing the stimulus frequency to 1.0 Hz, decreases in the overshoot potential, the maximum rate of rise and the action potential duration at 30%-repolarization level were much more apparent than at 0.5 Hz. The drug did not significantly modify the resting membrane potential and the total action potential duration. One of the experiments done with 0.5 Hz stimulation is shown in Fig. 1A, and summarized results are presented in Table 1.

Effects on slow response action potentials of canine ventricular muscles: Slow response action potentials were elicited at a rate of 0.1 Hz while the preparation was

Fig. 1. Effects of MCI-176 on normal action potentials and on slow response action potentials of canine ventricular muscles. **A:** normal action potentials recorded in 2.7 mM K^+ Tyrode's solution. **B:** slow response action potentials elicited in 24 mM K^+ Tyrode's solution in the presence of 10^{-6} M isoproterenol. Those were recorded with single cell impalements. **a:** control; **b–d:** 10^{-5} , 3×10^{-5} and 10^{-4} M MCI-176, respectively; **e:** superposition of **a–d**.

Table 1. Effects of MCI-176 on normal action potentials of the canine ventricular muscles

MCI-176	n	E_m (mV)	E_{ov} (mV)	E_p (mV)	V_{max} (V/sec)	APD ₃₀ (msec)	APD ₉₀ (msec)
0.5 Hz	7						
Control		-88 ± 2	22 ± 3	16 ± 2	166 ± 23	147 ± 13	253 ± 7
10^{-5} M		-87 ± 2	23 ± 3	18 ± 2	160 ± 20	144 ± 14	253 ± 8
3×10^{-5} M		-87 ± 2	22 ± 3	17 ± 2	155 ± 20^a	137 ± 14^a	253 ± 7
10^{-4} M		-88 ± 2	19 ± 3	14 ± 3	136 ± 17^a	129 ± 14^a	258 ± 10
1.0 Hz	6						
Control		-88 ± 1	24 ± 2	17 ± 2	127 ± 13	144 ± 11	253 ± 7
10^{-5} M		-88 ± 1	23 ± 2	16 ± 2	123 ± 13	144 ± 11	262 ± 9
3×10^{-5} M		-88 ± 1	21 ± 2^a	15 ± 3^a	107 ± 15^a	135 ± 9^a	259 ± 7
10^{-4} M		-87 ± 1	19 ± 2^a	14 ± 2^a	87 ± 14^a	124 ± 9^a	257 ± 6

n, number of preparations tested; E_m , resting membrane potential; E_{ov} , overshoot potential; E_p , plateau potential; V_{max} , maximum rate of rise of the action potential; APD₃₀ and APD₉₀, action potential duration at 30 and 90% repolarization level. Experiments were done with a single impalement. ^a significantly different from control values by the paired *t*-test at $P < 0.05$.

superfused with 27 mM K^+ Tyrode's solution containing 10^{-6} M isoproterenol. MCI-176 decreased both the overshoot potential and the action potential duration in a concentration-dependent manner at ranges from 10^{-5} to 10^{-4} M. One of the experiments is shown in Fig. 1B, and results are summarized in Table 2.

Effects on membrane currents of single ventricular cells of the guinea-pig: From the results obtained with canine ventricular muscles, it has strongly been suggested that MCI-176 antagonizes I_{Ca} . However, the

action potential duration and the plateau potential can be determined not only by I_{Ca} but also by the outward currents. Therefore, effects of MCI-176 on membrane currents were further studied in single ventricular cells using the "whole-cell" patch clamp method.

To evaluate effects of the drug on the inward calcium and outward potassium currents at different membrane potentials, ventricular cells were at first voltage clamped at the resting membrane potential level, at which no net outward and inward currents were observed, and 300 msec depolarizing

Table 2. Effects of MCI-176 on the slow response action potential induced by 27 mM K⁺ Tyrode's solution in the presence of 10⁻⁶ M isoproterenol

MCI-176	n	E _m (mV)	E _{ov} (mV)	APD ₅₀ (msec)	APD ₉₀ (msec)
Control	5	-40±1	22±3	90±14	107±14
10 ⁻⁶ M		-40±1	13±3 ^a	42±11 ^a	57±11 ^a
3×10 ⁻⁶ M		-40±1	4±4 ^a	22±6 ^a	32±8 ^a
10 ⁻⁴ M		-40±1	-3±4 ^a	17±4 ^a	26±6 ^a

Abbreviations, as in Table 1. Experiments were done with a single impalement.

Fig. 2. Effects of MCI-176 on membrane currents of a single ventricular cell of the guinea-pig. Membrane potential was held at the resting membrane potential, and membrane currents were obtained with the double-pulse protocol (see in the text). **A:** membrane currents obtained during voltage clamp from -30 mV to the depolarizing (a) or hyperpolarizing (superposed, b) potential listed on each trace. **B:** membrane currents obtained in the presence of 3×10⁻⁵ M MCI-176. **C:** current-voltage relations of the peak inward current (open symbols) and currents at the end of 300 msec clamp pulses (filled symbols) in the absence (circles) and in the presence (triangles) of the drug. Crosses indicate the current at the conditioning pulse of -30 mV. The dotted lines in A and B indicate the zero current level.

or hyperpolarizing test pulses which were preceded by the depolarizing conditioning pulse to -30 mV for 50 msec was applied every 10 sec (double-pulse protocol). Then the holding potential was changed to -30 mV, and the 300 msec depolarizing or hyperpolarizing test pulses were applied every 10

sec (single-pulse protocol). Following the control experiments, these pulse protocols were repeated in the presence of MCI-176. The peak amplitude of I_{Ca} obtained by the double pulse protocol was decreased with 3×10⁻⁵ M MCI-176 by 26% of the control value (Fig. 2). When the cell was held at -30

Fig. 3. Effects of MCI-176 (3×10^{-5} M) on membrane currents of a single ventricular cell of the guinea-pig. Membrane potential was held at -30 mV, and currents were obtained with the single-pulse protocol (see in the text). Crosses indicate the holding current at -30 mV. Other abbreviations are as in the Fig. 2 legend. All records in Figs. 2 and 3 were obtained from one and the same single cell.

mV, I_{Ca} was abolished by 3×10^{-5} M MCI-176 (Fig. 3). Outward currents between -75 and -30 mV were decreased by the drug (Figs. 2 and 3). Similar results were obtained in other 4 cells.

To avoid influences of the run-down of I_{Ca} , the order of the holding potential was randomized, and also effects of the drug were examined at only one holding potential in other series of experiments. Those results were qualitatively the same as the results shown in Figs. 2 and 3; that is, the depression of I_{Ca} by MCI-176 was holding potential-dependent and was much more apparent in the less negative holding potential.

Effects on the isolated I_{Ca} : In order to minimize influences of the outward potassium currents, the potassium currents were abolished by cesium ions. Ventricular cells were dialyzed internally with the Cs-internal pipette solution through the patch electrode and were superfused with Cs-Tyrodé's solution. Under this circumstance, inward and

outward potassium currents were almost abolished within 4–6 min (7). Then, the effect of MCI-176 on the isolated I_{Ca} which was elicited by the double-pulse protocol was examined. Membrane potentials were held at -60 mV, and test 300 msec depolarizing pulses were preceded by conditioning pulses to -30 mV for 50 msec. As shown in Fig. 4, the isolated I_{Ca} reached a maximum value at $+10$ mV, and the apparent reversal potential obtained by extrapolating the current-voltage relationship curve was approximately $+75$ mV.

The isolated I_{Ca} was clearly decreased by 3×10^{-5} MCI-176 without affecting the apparent reversal potential.

Use- and frequency-dependent effects of MCI-176 on I_{Ca} : Use-dependency of the effect of MCI-176 on I_{Ca} was examined by the following experimental protocol. I_{Ca} was elicited by 200 msec depolarizing test pulses to 0 mV while membrane potentials were held at -30 mV (single pulse protocol). The test

Fig. 4. Effects of MCI-176 on the isolated inward calcium current (isolated I_{Ca}). Membrane potential was held at -60 mV, and membrane currents were recorded with the double-pulse protocol in the Cs/Cs condition (see in the text). A: superposed membrane currents obtained during voltage clamp from -30 mV to 50 mV by a 10 -mV step in the absence (a) and in the presence (b) of 3×10^{-5} M MCI-176. The dotted lines in A indicate the zero current level. B: current-voltage relations of the amplitude of the isolated I_{Ca} in the absence (circles) and in the presence (triangles) of the drug.

Fig. 5. Use-dependent effect of MCI-176 on the amplitude of I_{Ca} induced by repetitive depolarization after a 5-min rest. Membrane potential was held at -30 mV, and the cell was stimulated at 3-sec interval in the absence (open circles) and in the presence (open triangles) of 10^{-6} M MCI-176.

pulse was applied every 3 sec. For the control, depolarizing test pulses were applied following a 5-min quiescent period until a steady-state amplitude of I_{Ca} was obtained.

Then, depolarizing pulses were discontinued and the drug was applied. Depolarizing test pulses were resumed after a 5-min rest in the presence of the drug. To avoid the complete

Fig. 6. Frequency-dependent effect of MCI-176 (10^{-5} M) on the relative amplitude of I_{Ca} . Membrane potential was held at the resting membrane potential, and I_{Ca} was obtained by the double-pulse protocol in the absence (open circles) and in the presence (open triangles) of MCI-176. Amplitudes were expressed as relative to that of 10-sec interval stimulation.

abolition of I_{Ca} by the initial block, a relatively low concentration of MCI-176 was used in this experiment. As shown in Fig. 5, the reduction of the amplitude of I_{Ca} comparing the first and the 10th amplitude was 32% in the absence of the drug. When 10^{-6} M MCI-176 was applied under resting conditions, the first test pulse elicited the attenuated I_{Ca} ; this "initial block" amounted to 46%, and the reduction of the amplitude comparing the first and the 10th amplitude was 90%.

Frequency-dependence was taken as synonymous to use-dependency. The effects of MCI-176 on the steady-state amplitude of I_{Ca} were also evaluated at different stimulus intervals. I_{Ca} was elicited by the double-pulse protocol. Membrane potentials were held at the resting membrane potential and 200 msec depolarizing test pulses to 0 mV were applied following the 50 msec conditioning pulse to -30 mV. The stimulus interval was changed from 10 to 5, 3, 2, 1 and 0.5 sec. As shown in Fig. 6, in the drug free condition, a reduction of the amplitude was observed from 3-sec interval stimulation, and it amounted to 49% of the control (10-sec) value at 0.5-sec interval stimulation. In the presence of 10^{-5} M

MCI-176, the reduction of the amplitude induced by a decrease in the stimulus interval was accelerated and I_{Ca} was almost abolished at 0.5-sec interval. The relative difference between the predrug and the drug affected values was 18, 40, 54, 59 and 49% of the control (10-sec) at 5-, 3-, 2-, 1- and 0.5-sec interval, respectively. That is, the reduction of the amplitude of I_{Ca} induced by shortening the stimulus interval was extremely augmented by MCI-176.

Discussion

In the present experiments with canine ventricles, MCI-176 (3×10^{-5} and 10^{-4} M) decreased the action potential duration at 30%-repolarization level and the maximum rate of rise of the action potential in a concentration-dependent manner. These effects appeared to be stimulus frequency-dependent. The amplitude and duration of the slow response action potential was also decreased by the drug in a concentration-dependent manner (10^{-5} – 10^{-4} M). MCI-176 has been reported to be slightly more potent than diltiazem on a weight basis in producing coronary vasodilation in anesthetized dogs

(4). Furthermore, a previous study (5) has demonstrated that MCI-176 has a cardiovascular profile common to non-vasoselective calcium antagonists like diltiazem and verapamil. The lower effectiveness in depressing the force of contraction than diltiazem and verapamil is a characteristic of this drug. Reduction of the maximum rate of rise also been reported with verapamil (9), D600 (10) and diltiazem (11), and this could be interpreted as a non-specific suppression of the sodium current. In an article on the electrophysiological effects of diltiazem, Nakajima et al. (11) have described that the maximum rate of rise of the action potential was first depressed with 2.2×10^{-5} M without changing other action potential parameters, and the duration of action potential was shortened with 1.1×10^{-4} M. They have also studied the effect of diltiazem on slow response action potentials (12). The amplitude and duration of slow response action potentials were first affected with 1.1×10^{-5} M diltiazem. Thus, there is only a 2-fold difference on threshold concentrations of diltiazem affecting the slow calcium current and the fast sodium current. Nevertheless, diltiazem has been classified as a "specific calcium antagonist" (13). Indeed, diltiazem does not affect impulse conduction through the His-Purkinje-ventricular system where the fast sodium current plays a decisive role in conduction when diltiazem is injected into the anterior septal artery which supplies the His-Purkinje-ventricular system. Like diltiazem, MCI-176 does not affect impulse conduction through the His-Purkinje-ventricular system (5). Furthermore, MCI-176 is rather ineffective in suppressing the force of contraction of canine papillary muscle. Therefore, it might be quite reasonable to conclude that MCI-176 is a "specific calcium antagonist" similar to diltiazem.

The action potential duration and the plateau potential, however, can be determined not only by I_{Ca} but also by the outward currents, and this is also true with the slow response action potential (14). Therefore, the effects of the drug were analyzed with voltage clamp experiments using single ventricular cells of the guinea-pig. It has been reported that calcium antagonists act on I_{Ca}

in a voltage- and use-dependent manner (15–19). In the present experiments, the effects of MCI-176 were examined at two different holding potentials, the resting membrane potential (-70 – -80 mV) and -30 mV. When the membrane potential was held at the resting potential, a 50-msec conditioning pulse to -30 mV preceded the test pulse in order to inactivate the sodium current. Blockade of I_{Ca} was more pronounced in the less negative holding potential; that is, the voltage-dependent block of I_{Ca} was clearly demonstrated (Figs. 2 and 3). This indicates that MCI-176 has a strong affinity to the inactivated state of calcium channels like other calcium antagonists (16, 20, 21). Selective blockade of I_{Ca} was confirmed by the experiments under the Cs/Cs condition in which potassium currents were minimized (Fig. 4). Use- and frequency-dependent blocks of I_{Ca} by MCI-176 were also examined. When stimulus intervals were fixed at 3 sec, following the 5-min rest, the amplitude of I_{Ca} was gradually decreased and reached a steady state value within 1–1.5 min (Fig. 5, open circles). Then MCI-176 was applied at rest, and after the 5-min rest, the depolarizing test pulses were resumed. In the presence of the drug, I_{Ca} was almost abolished by 3–4 depolarizing pulses (Fig. 5, open triangles). It has been reported that verapamil and D600 produce very little inhibition in the absence of test pulses, but blockade increases with repeated depolarizations (15–17, 22). The cumulative effect is extremely small with nitrendipine, and diltiazem is intermediate (17). MCI-176 suppressed the very first current following the rest, and the blockade increased with repeated depolarizations (Fig. 5). This kind of blocking nature was quite similar to the result reported with diltiazem (17, 18). The amplitude of I_{Ca} was decreased by increasing stimulus interval probably because of its slow recovery from inactivation. Suppression by the drug of I_{Ca} was much more pronounced at a higher stimulus frequency (Fig. 6). This agrees well with results obtained in the canine ventricular muscles (Fig. 1 and Table 1). It has been reported that MCI-176 increases the coronary blood flow and decreases mean arterial pressure (4), and it decreases the sino-atrial rate and increases

the atrio-ventricular conduction time (5). These can be explained by the calcium channel blocking action of this drug.

From these results, it can be concluded that MCI-176 decreases I_{Ca} of the mammalian ventricular muscle cells, and the effect is membrane voltage- and use-dependent.

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