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Cardioprotective effect of MCC-135 is associated with inhibition of Ca^{2+} overload in ischemic/reperfused hearts

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Abstract

Calcium (Ca^{2+}) overload is an important pathophysiological factor in myocardial ischemic/reperfusion injury. We investigated the effects of a cardioprotective drug, MCC-135, 5-methyl-2-(1-piperazinyl) benzenesulfonic acid monohydrate, on (1) cardiac contractile dysfunction and Ca^{2+} overload induced by ischemia and reperfusion, and (2) the Na^+/Ca^{2+} exchanger in Langendorff-perfused rat hearts. Low-flow 45-min ischemia and 30-min reperfusion decreased developed tension and increased ventricular Ca^{2+} content, effects which were ameliorated by MCC-135 and amiloride given after reperfusion. Combination of intracellular Na^+ overload induced by monensin (Na^+ ionophore; 5 μM) and zero-flow 15-min ischemia followed by 30-min reperfusion resulted in a decrease in developed tension and in the intracellular Na^+-dependent increase in ventricular Ca^{2+} content. MCC-135 and the highest dose of amiloride given after reperfusion reduced the increase in ventricular Ca^{2+} content, whereas developed tension was increased only with MCC-135. These results suggest that the cardioprotective effect of MCC-135 in ischemia/reperfusion is associated with suppression of Ca^{2+} overload and is attributable to inhibition of intracellular Na^+-dependent Ca^{2+} influx via the Na^+/Ca^{2+} exchanger.

Keywords: MCC-135; Cardioprotection; Ischemia; Reperfusion; Ca^{2+} overload

1. Introduction

The rationale for reperfusing the myocardium as early as possible is to salvage the ischemic myocardium. This has been clinically made possible by the development of revascularization techniques, such as percutaneous coronary intervention and thrombolytic therapy (Weaver et al., 1997). However, reperfusion itself may cause additional tissue damage referred to as "reperfusion injury" (Braunwald and Kloner, 1985). It has been suggested that myocardial calcium (Ca^{2+}) overload is, at least in part, responsible for reperfusion injury (Kusuoka et al., 1987).

During ischemia, the breakdown of ATP and the production of lactate result in intracellular acidosis (Piper et al., 1996), which could in turn stimulate the Na^+/H^+ exchanger to regulate intracellular pH. The activity of the Na^+/H^+ exchanger, however, is reduced because extracellular pH is decreased secondary to intracellular acidosis while no-flow ischemia persists (Piper et al., 1996). A rapid washout of extracellular H^+ upon reperfusion reactivates the Na^+/H^+ exchanger and causes intracellular Na^+ overload (Pike et al., 1993), which may facilitate the subsequent influx of Ca^{2+} via the Na^+/Ca^{2+} exchanger, causing myocardial Ca^{2+} overload (Van Emous et al., 1998). The myocardial Ca^{2+} overload induced by reperfusion is an important pathophysiological factor that contributes to mechanical dysfunction, myocardial cell death and arrhythmia (Kusuoka et al., 1987; Silverman and Stern, 1994; Piper et al., 2003). Therefore, pharmacological interventions that inhibit myocardial Ca^{2+} overload could be beneficial in ameliorating reperfusion injury.

In fact, inhibition of Ca^{2+} overload subsequent to Na^+/H^+ exchanger inhibition (Rupprecht et al., 2000; Stromer et al., 2000; Kusumoto et al., 2002) or Na^+/Ca^{2+} exchanger inhibition (Seki et al., 2002; Elias et al., 2001) has been

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demonstrated to improve myocardial function, to decrease the incidence of arrhythmia and to limit infarct size during ischemia and reperfusion. However, inhibition of L-type Ca^{2+} channels, another potential pathway of Ca^{2+} influx, failed to reduce myocardial injury when Ca^{2+} channel blockade was initiated at the time of reperfusion (Inagaki et al., 2000).

MCC-135, 5-methyl-2-(1-piperazinyl) benzenesulfonic acid monohydrate, is a new drug with cardioprotective effects in ischemia and reperfusion. In anesthetized dogs subjected to regional myocardial ischemia and reperfusion, MCC-135 improved regional myocardial mechanical dysfunction (Kawasumi et al., 1999) and reduced myocardial infarct size (Kawasumi et al., 1998) when administered just before reperfusion. Recently, MCC-135 was demonstrated to improve regional contractility and to reduce the plasma creatine kinase level in pigs in which the left circumflex coronary artery was occluded and released (Yarbrough et al., 2003). However, the mechanism of the cardioprotective effects of MCC-135, which has no affinity for L-type Ca^{2+} channels and β-adrenoceptors, remains to be studied. In this study, we investigated the effects of MCC-135 on cardiac contractile dysfunction and myocardial Ca^{2+} overload in isolated perfused rat hearts subjected to ischemia and reperfusion with and without previous intracellular Na^+ overload.

2. Materials and methods

The experimental protocols in this study were approved by the Animal Care and Use Committee of Mitsubishi Pharma Corporation.

2.1. Preparation of Langendorff-perfused hearts

Male Sprague–Dawley rats (250–350 g) were anesthetized with pentobarbital sodium (50 mg/kg i.p.). The heart was excised and cannulated through the aorta, and perfused with Krebs buffer solution (in mM: NaCl 119, KCl 4.6, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 2H_2O$ 1.3, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11; pH 7.4, 37 °C) aerated with 95% O_2+5% CO_2 according to the Langendorff method. The temperature of the heart was maintained at 37 °C by a surrounding water-heated jacket. The perfusion pressure was maintained at 80 mm Hg. For the measurement of developed tension, a thread attached to the apex of the heart was connected to a strain-gauge tension transducer (NEC Medical Systems UL-10GR, Tokyo, Japan). The heart was equilibrated for 1 h before experiments. During the equilibration period, the position of the transducer was gradually adjusted to yield a diastolic tension of approximately 1 g.

2.2. Experimental protocol

The perfusion protocols used in this study are summarized in Fig. 1.

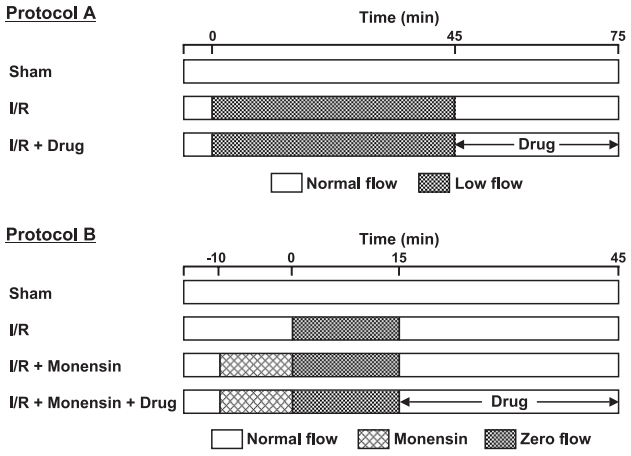

Fig. 1. Perfusion protocols. Protocol A was used to determine the effects of drugs on cardiac contractile dysfunction and Ca^{2+} overload induced by low-flow ischemia (45 min) and reperfusion (30 min). Protocol B was used to determine the effects of drugs on cardiac contractile dysfunction and Ca^{2+} overload induced by pretreatment with monensin (5 μM, 10 min) followed by zero-flow ischemia (15 min) and reperfusion (30 min). I/R, ischemia and reperfusion.

2.2.1. Ischemia and reperfusion (Protocol A)

Following baseline perfusion, myocardial ischemia was induced by perfusing the heart with modified Krebs buffer solution (in mM: NaCl 119, KCl 4.6, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 2H_2O$ 1.3, $NaHCO_3$ 25, KH_2PO_4 1.2, sucrose 11; pH 7.4, 37 °C) aerated with 95% O_2+5% CO_2 at a perfusion pressure of 5 mm Hg for 45 min. The heart was then reperfused with normal Krebs buffer solution at the original perfusion pressure of 80 mm Hg for 30 min. To test the effects of drugs, the drugs were present in the reperfusate only, with only one drug dose being tested in each heart. In the sham group, perfusion with normal Krebs buffer solution at a perfusion pressure of 80 mm Hg was continued throughout the experiment. The number of hearts used in each group was 8 to 10.

2.2.2. Ischemia and reperfusion with monensin-pretreatment (Protocol B)

Following baseline perfusion, the heart was perfused with normal Krebs buffer solution containing monensin (5 μM) for 10 min and then subjected to zero-flow ischemia by clamping the coronary flow for 15 min. The heart was then reperfused with normal Krebs buffer solution at the original perfusion pressure of 80 mm Hg for 30 min. To test the effects of drugs, the drugs were present in the reperfusate only, with only one drug dose being tested in each heart. This protocol of combined monensin pretreatment and zero-flow ischemia has been demonstrated to cause intracellular Na^+ overload, which was followed by Ca^{2+} overload mediated by the Na^+/Ca^{2+} exchanger upon reperfusion (Tani and Neely, 1989). The number of hearts used in each group was 8.

2.3. Measurement of myocardial Ca^{2+} and Na^{+} content

After completion of the perfusion protocol, the coronary artery was flushed with 10 ml of cold, deionized flushing solution (0.35 M sucrose, 5 mM histidine, pH 7.4) treated with Dowex (50W-X2, BioRad). This procedure has been shown to wash out more than 90% of extracellular ions, thereby minimizing the contribution of extracellular Ca^{2+} and Na^{+} to the measured Ca^{2+} and Na^{+} (Tosaki et al., 1990). This flushing technique has been described in normoxic hearts (Alto and Dhalla, 1979), anoxic hearts (Fiolet et al., 1984) and ischemic/reperfused hearts (Pridjian et al., 1987; Nayler et al., 1988). Thus, the Ca^{2+} and Na^{+} content determined by our method can be considered as myocardial Ca^{2+} and Na^{+}. Methods based on the use of extracellular markers to determine extracellular space were avoided because of their inherent unreliability when cellular permeability is altered by ischemia and reperfusion (Nayler et al., 1984). The atria and the large coronary arteries were discarded and the ventricle was blotted, weighed, and then dried to constant weight at 100 °C. The dried ventricle was weighed and digested in concentrated HNO_3 for 18 h at 130 °C. The sample solution was supplemented with lanthanum chloride after sufficient cooling. The Ca^{2+} and Na^{+} contents were determined from optical density measurements recorded at 422.7 and 589.0 nm, respectively, using an atomic absorption spectrometer (Z-6000, Hitachi, Japan). The myocardial Ca^{2+} and Na^{+} contents are expressed as micromoles per gram ventricular dry weight.

2.4. Chemicals

MCC-135, 5-methyl-2-(1-piperazinyl) benzenesulfonic acid monohydrate, was synthesized by Mitsubishi Pharma. Diltiazem and amiloride were purchased from Sigma (St. Louis, MO, USA). All drugs were directly dissolved in Krebs buffer solution.

2.5. Statistical analysis

The results are presented as means±S.E.M. When two groups were compared, unpaired Student's *t*-test or Welch's test was used. When values at baseline and after monensin treatment were compared, paired Student's *t*-test was used. When more than two groups were compared, one-way analysis of variance followed by Dunnett's test or Kruskal Wallis test followed by Dunn's test were used. Differences were considered significant at a value of $P<0.05$.

3. Results

3.1. Ischemia and reperfusion (Protocol A)

Systolic tension, diastolic tension, developed tension and heart rate at baseline, after ischemia (45 min of ischemia) and after reperfusion (30 min of reperfusion) in sham and ischemia/reperfusion groups are presented in Table 1. There were no significant differences in any of these parameters between the groups at baseline. Diastolic tension after ischemia was significantly higher in the ischemia/reperfusion group than in the sham group ($P<0.001$). After reperfusion, systolic tension ($P<0.05$) and developed tension ($P<0.05$) were lower, and diastolic tension was higher ($P<0.05$) in the ischemia/reperfusion group than in the sham group. Fig. 2A shows the recovery of developed tension after 30 min of reperfusion, expressed as a percentage of baseline developed tension. Recovery of developed tension was significantly lower in the ischemia/reperfusion group than in the sham group (25.0±3.4% vs. 89.6±2.0%, $P<0.001$). Fig. 2B shows the ventricular contents of Ca^{2+} and Na^{+} normalized to the ventricular dry weight. Both the Ca^{2+} and Na^{+} contents of the ventricle were significantly higher in the ischemia/reperfusion group than in the sham group (Ca^{2+} 3.61±0.22 vs. 2.19±0.11 μmol/g, $P<0.001$; Na^{+} 79.6±5.1 vs. 59.4±4.6 μmol/g, $P<0.01$).

Systolic tension, diastolic tension, developed tension and heart rate at baseline, ischemia (45 min of ischemia) and reperfusion (30 min of reperfusion) in non-treated and drug-treated groups are presented in Table 2. There were no significant differences in any of these parameters among the groups at baseline and ischemia. Since the drugs were applied after reperfusion, the lack of statistical significance in diastolic tension at the end of ischemia among the groups suggests that ischemic injury was similar in each group. MCC-135 increased systolic tension ($P<0.01$ at 10^{-8}, 10^{-7}, 10^{-6} M) and developed tension ($P<0.05$ at 10^{-7} M, $P<0.01$ at 10^{-6} M) at reperfusion. Diltiazem ($P<0.05$ at 10^{-6} and 10^{-5} M) and amiloride ($P<0.01$ at 10^{-3} M) decreased the

Table 1
Tension and heart rate in a protocol of ischemia and reperfusion (protocol A)

	Baseline	Ischemia	Reperfusion
Systolic tension (g)			
Sham	3.61±0.50	3.38±0.49	3.30±0.49
I/R	4.59±0.52	6.31±0.77[a]	2.06±0.16[b]
Diastolic tension (g)			
Sham	1.01±0.01	0.96±0.02	0.94±0.02
I/R	0.99±0.01	6.31±0.77[c]	1.20±0.09[b]
Developed tension (g)			
Sham	2.59±0.49	2.42±0.49	2.35±0.48
I/R	3.60±0.52	0.0	0.86±0.13[b]
Heart rate (beats/min)			
Sham	289±19	278±17	275±18
I/R	325±19	0	312±25

The hearts were subjected to low-flow ischemia (45 min) and reperfusion (30 min). Values are means±S.E.M. for 9–10 separate hearts. I/R, ischemia and reperfusion.

[a] $P<0.01$ vs. sham.
[b] $P<0.05$ vs. sham.
[c] $P<0.001$ vs. sham.

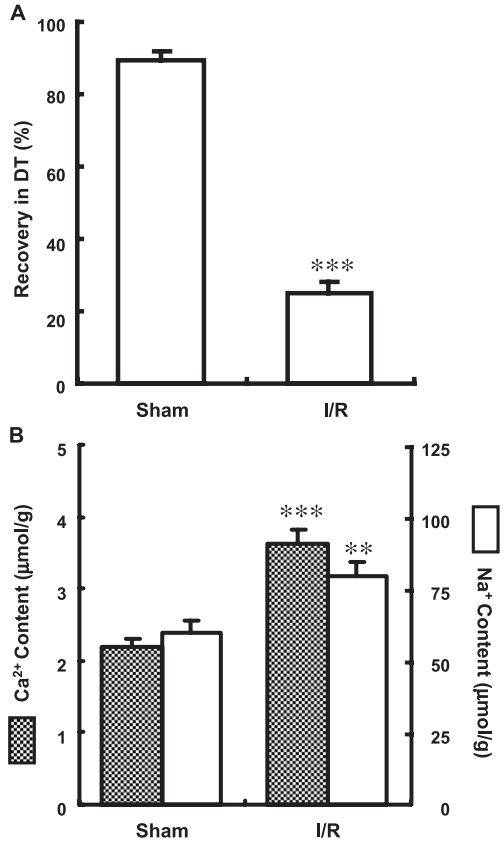

Fig. 2. Recovery of developed tension, expressed as a percentage of baseline developed tension, (A) and ventricular Ca^{2+} and Na^{+} contents normalized to ventricular dry weight (B) after 30 min of reperfusion following 45 min of low-flow ischemia (protocol A) in sham and ischemia/reperfusion groups. Values are means±S.E.M. for 9–10 separate hearts. $^{**}P<0.01$, $^{***}P<0.001$ vs. sham. DT, developed tension; I/R, ischemia and reperfusion.

heart rate at reperfusion. Fig. 3 shows the recovery of developed tension after 30 min of reperfusion, expressed as a percentage of baseline developed tension (top), and the ventricular contents of Ca^{2+} and Na^{+} normalized to the ventricular dry weight (bottom) in non-treated and drug-treated groups. MCC-135 increased the recovery of developed tension ($P<0.01$ at 10^{-7} M, $P<0.001$ at 10^{-6} M). Reciprocally, MCC-135 decreased the ventricular content of Ca^{2+} ($P<0.001$ at 10^{-7} and 10^{-6} M) and Na^{+} ($P<0.05$ at 10^{-6} M). Amiloride, an inhibitor of the Na^{+}/H^{+} exchanger, increased the recovery of developed tension ($P<0.05$ at 10^{-3} M), accompanied by a decrease in Ca^{2+} content ($P<0.001$ at 10^{-3} M). Diltiazem, a Ca^{2+} channel antagonist, further decreased the recovery of developed tension to below the non-treated level without significantly affecting either the Ca^{2+} or the Na^{+} content.

3.2. Ischemia and reperfusion with monensin pretreatment (Protocol B)

Systolic tension, diastolic tension, developed tension and heart rate at baseline and after monensin treatment (10 min of monensin treatment), ischemia (15 min of ischemia) and reperfusion (30 min of reperfusion) in the sham, ischemia/reperfusion and ischemia/reperfusion pretreated with monensin groups are presented in Table 3. There were no

Table 2
Tension and heart rate in a protocol of ischemia and reperfusion (protocol A)

	Baseline	Ischemia	Reperfusion
Systolic tension (g)			
NT	4.59±0.52	6.31±0.77	2.06±0.16
MCC-135 10^{-9} M	4.89±0.74	6.14±0.68	2.72±0.36
MCC-135 10^{-8} M	5.30±0.53	7.10±0.69	3.27±0.34[a]
MCC-135 10^{-7} M	4.42±0.43	5.96±0.44	3.19±0.29[a]
MCC-135 10^{-6} M	4.05±0.19	5.39±0.76	3.28±0.18[a]
Diltiazem 10^{-7} M	3.78±0.26	5.20±0.42	2.07±0.22
Diltiazem 10^{-6} M	4.50±0.45	5.33±0.47	1.90±0.13
Diltiazem 10^{-5} M	3.75±0.32	4.95±0.54	1.61±0.22
Amiloride 10^{-5} M	4.40±0.47	4.89±0.47	2.52±0.27
Amiloride 10^{-4} M	4.16±0.29	5.13±0.46	2.57±0.19
Amiloride 10^{-3} M	3.78±0.27	4.91±0.39	2.55±0.12
Diastolic tension (g)			
NT	0.99±0.01	6.31±0.77	1.20±0.09
MCC-135 10^{-9} M	1.04±0.03	6.14±0.68	1.12±0.08
MCC-135 10^{-8} M	1.01±0.01	7.10±0.69	1.15±0.07
MCC-135 10^{-7} M	0.98±0.03	5.96±0.44	0.88±0.07
MCC-135 10^{-6} M	1.04±0.05	5.39±0.76	0.91±0.06
Diltiazem 10^{-7} M	1.01±0.02	5.20±0.42	1.15±0.13
Diltiazem 10^{-6} M	1.02±0.01	5.33±0.47	1.44±0.12
Diltiazem 10^{-5} M	1.02±0.02	4.95±0.54	1.46±0.21
Amiloride 10^{-5} M	1.04±0.02	4.89±0.47	1.42±0.21
Amiloride 10^{-4} M	0.97±0.02	5.13±0.46	1.08±0.05
Amiloride 10^{-3} M	1.02±0.01	4.91±0.39	0.96±0.08
Developed tension (g)			
NT	3.60±0.52	0.0	0.86±0.13
MCC-135 10^{-9} M	3.85±0.76	0.0	1.61±0.37
MCC-135 10^{-8} M	4.29±0.54	0.0	2.12±0.29
MCC-135 10^{-7} M	3.44±0.43	0.0	2.31±0.26[b]
MCC-135 10^{-6} M	3.01±0.21	0.0	2.37±0.18[a]
Diltiazem 10^{-7} M	2.77±0.27	0.0	0.93±0.23
Diltiazem 10^{-6} M	3.48±0.45	0.0	0.47±0.13
Diltiazem 10^{-5} M	2.73±0.33	0.0	0.15±0.02
Amiloride 10^{-5} M	3.36±0.45	0.0	1.10±0.24
Amiloride 10^{-4} M	3.19±0.29	0.0	1.49±0.22
Amiloride 10^{-3} M	2.76±0.27	0.0	1.59±0.17
Heart rate (beats/min)			
NT	325±19	0	312±25
MCC-135 10^{-9} M	343±16	0	325±29
MCC-135 10^{-8} M	314±15	0	300±13
MCC-135 10^{-7} M	301±16	0	273±17
MCC-135 10^{-6} M	341±15	0	300±15
Diltiazem 10^{-7} M	349±16	0	302±24
Diltiazem 10^{-6} M	278±11	0	204±41[b]
Diltiazem 10^{-5} M	334±14	0	207±32[b]
Amiloride 10^{-5} M	314±16	0	291±28
Amiloride 10^{-4} M	308±12	0	254±15
Amiloride 10^{-3} M	332±18	0	133±34[a]

The hearts were subjected to low-flow ischemia (45 min) and reperfusion (30 min). Drugs were present only during reperfusion. Values are means±S.E.M for 8–10 separate hearts. NT, non-treatment.

[a] $P<0.01$ vs. sham.

[b] $P<0.05$ vs. sham.

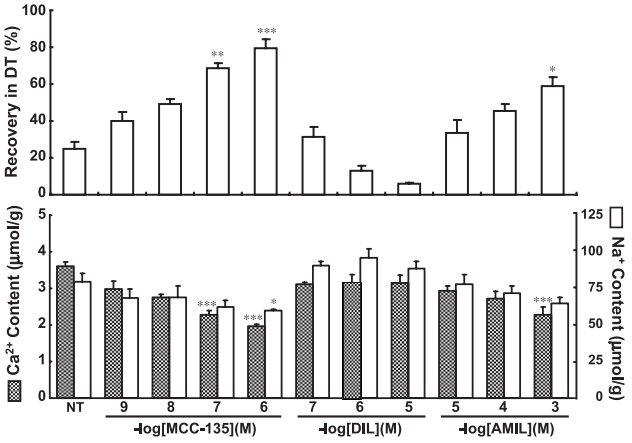

Fig. 3. Effects of MCC-135, diltiazem (DIL) and amiloride (AMIL) on recovery of developed tension, expressed as a percentage of baseline developed tension, (top panel) and ventricular Ca^{2+} and Na^{+} contents normalized to ventricular dry weight (bottom panel) after 30 min of reperfusion following 45 min of low-flow ischemia (protocol A). Drugs were present only during reperfusion. Values are means±S.E.M. for 8–10 separate hearts. *P<0.05, **P<0.01, ***P<0.001 vs. non-treatment (NT). DT, developed tension.

significant differences in any of these parameters among the groups at baseline, except for diastolic tension in the ischemia/reperfusion pretreated with monensin group compared with the sham group (1.05±0.01 vs. 1.00±0.01 g, P<0.05). Monensin treatment for 10 min, prior to ischemia, significantly increased the heart rate compared with baseline (382±11 vs. 309±22 beats/min, P<0.05), without significantly affecting systolic tension, diastolic tension and developed tension. After ischemia, diastolic tension was significantly higher in the ischemia/reperfusion group (P<0.05) and in the ischemia/reperfusion pretreated with monensin group (P<0.001) than in the sham group. After reperfusion, developed tension (P<0.001) and heart rate (P<0.01) were lower, and diastolic tension was higher (P<0.001), in the ischemia/reperfusion treated with monensin group than in the sham group. Fig. 4A shows the recovery of developed tension after 30 min of reperfusion, expressed as a percentage of baseline developed tension. The recovery of developed tension was significantly lower in the ischemia/reperfusion pretreated with monensin group than in the sham group (5.3±2.1% vs. 91.2±2.9%, P<0.001). Fig. 4B shows the ventricular contents of Ca^{2+} and Na^{+} normalized to the ventricular dry weight. Both the Ca^{2+} and Na^{+} contents in the ventricle were significantly higher in the ischemia/reperfusion pretreated with monensin

Table 3
Tension and heart rate in a protocol of ischemia and reperfusion with monensin pretreatment (protocol B)

	Baseline	Monensin	Ischemia	Reperfusion
Systolic tension (g)				
Sham	4.13±0.34	3.38±0.26	3.94±0.31	3.76±0.29
I/R	4.89±0.26	4.26±0.22	3.01±0.54	3.44±0.11
I/R+Monensin	4.87±0.41	4.55±0.41[a]	5.15±0.92	4.16±0.64
Diastolic tension (g)				
Sham	1.00±0.01	1.02±0.02	0.93±0.03	0.91±0.03
I/R	1.05±0.02	1.04±0.05	3.01±0.54[a]	0.99±0.07
I/R+Monensin	1.05±0.01[a]	1.23±0.14	5.15±0.92[b]	3.98±0.66[b]
Developed tension (g)				
Sham	3.14±0.34	2.36±0.27	3.01±0.32	2.84±0.29
I/R	3.84±0.26	3.22±0.23	0	2.44±0.16
I/R+Monensin	3.82±0.40	3.33±0.40	0	0.19±0.07[b]
Heart rate (beats/min)				
Sham	328±16	348±10	317±17	324±14
I/R	307±16	335±16	0	311±16
I/R+Monensin	309±22	382±11[c]	0	172±41[d]

The hearts were subjected to pretreatment with monensin (5 μM, 10 min), zero-flow ischemia (15 min) and reperfusion (30 min). Values are means±S.E.M. for 8 separate hearts. I/R, ischemia and reperfusion.

[a] P<0.05 vs. sham.
[b] P<0.001 vs. sham.
[c] P<0.05 vs. baseline.
[d] P<0.01 vs. sham.

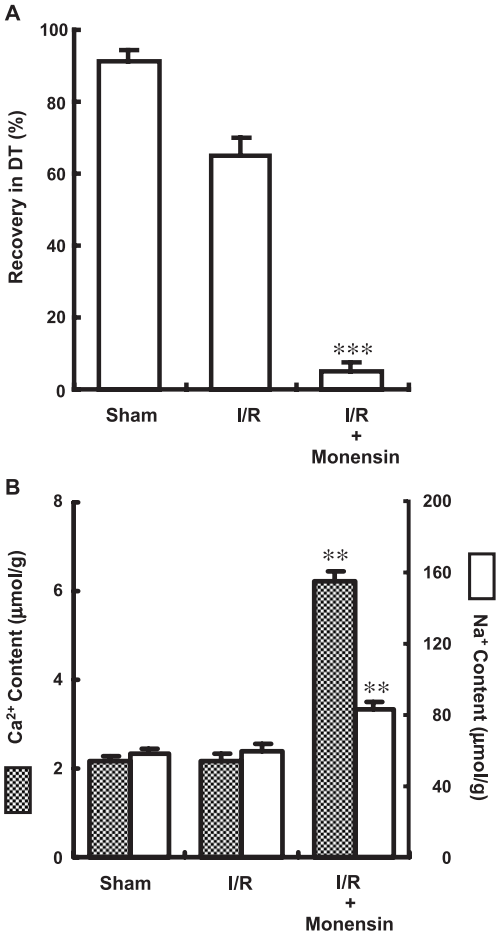

Fig. 4. Recovery of developed tension, expressed as a percentage of baseline developed tension, (A) and ventricular Ca^{2+} and Na^{+} contents normalized to ventricular dry weight (B) at 30 min of reperfusion following monensin pretreatment (5 μM, 10 min) and 15 min of zero-flow ischemia (protocol B) in sham, ischemia/reperfusion and monensin-pretreated ischemia/reperfusion groups. Values are means±S.E.M. for 8 separate hearts. **P<0.01, ***P<0.001 vs. sham. DT, developed tension; I/R, ischemia and reperfusion.

group than in the sham group (Ca^{2+} 6.20±0.23 vs. 2.15±0.11 μmol/g, $P<0.01$; Na^{+} 83.4±4.5 vs. 57.9±3.5 μmol/g, $P<0.01$).

Systolic tension, diastolic tension, developed tension and heart rate at baseline and after monensin treatment (10 min of monensin treatment), ischemia (15 min of ischemia) and reperfusion (30 min of reperfusion) in non-treated and drug-treated groups are presented in Table 4. There were no significant differences in any of these parameters at baseline, or after monensin treatment and ischemia among the groups. Since the drugs were applied after reperfusion, the lack of statistical significance in diastolic tension at ischemia among the groups suggests that ischemic injury was similar in each group. After reperfusion, MCC-135 increased developed tension ($P<0.01$ at 10^{-7} M, $P<0.001$ at 10^{-6} M) and decreased diastolic tension ($P<0.05$ at 10^{-6} M). Fig. 5 shows the developed tension after 30 min of reperfusion, expressed as a percentage of baseline developed tension (top), and the ventricular contents of Ca^{2+} and Na^{+} normalized to the ventricular dry weight (bottom) in non-treated and drug-treated groups. MCC-135 increased the recovery of developed tension ($P<0.01$ at 10^{-7} M, $P<0.001$ at 10^{-6} M), accompanied by a decrease in ventricular Ca^{2+} content ($P<0.01$ at 10^{-7} and 10^{-6} M). Amiloride decreased the ventricular Ca^{2+} content ($P<0.01$ at 10^{-3} M) without significantly affecting the recovery of developed tension. Diltiazem had no significant effects on the recovery of developed tension or on the ventricular contents of Ca^{2+} or Na^{+}.

Table 4
Tension and heart rate in a protocol of ischemia and reperfusion with monensin pretreatment (protocol B)

	Baseline	Monensin	Ischemia	Reperfusion
Systolic tension (g)				
NT	4.87±0.41	4.55±0.41	5.15±0.92	4.16±0.64
MCC-135 10^{-9} M	4.88±0.41	4.79±0.52	6.38±0.68	4.88±0.52
MCC-135 10^{-8} M	4.44±0.25	4.30±0.35	4.28±0.67	3.74±0.43
MCC-135 10^{-7} M	4.51±0.42	4.34±0.35	4.62±0.70	3.55±0.46
MCC-135 10^{-6} M	4.36±0.34	3.75±0.30	4.25±0.79	3.44±0.21
Diltiazem 10^{-6} M	3.90±0.38	3.34±0.35	4.08±0.58	3.00±0.37
Diltiazem 10^{-5} M	5.48±0.42	4.57±0.24	4.43±0.76	3.29±0.31
Amiloride 10^{-4} M	4.18±0.31	3.83±0.37	4.99±0.48	3.81±0.39
Amiloride 10^{-3} M	4.44±0.29	4.09±0.38	3.97±0.51	3.56±0.33
Diastolic tension (g)				
NT	1.05±0.01	1.23±0.14	5.15±0.92	3.98±0.66
MCC-135 10^{-9} M	1.05±0.03	1.25±0.14	6.38±0.68	4.53±0.52
MCC-135 10^{-8} M	1.02±0.02	1.03±0.03	4.28±0.67	3.14±0.43
MCC-135 10^{-7} M	1.03±0.03	1.05±0.06	4.62±0.70	2.74±0.46
MCC-135 10^{-6} M	1.03±0.01	1.10±0.08	4.25±0.79	2.20±0.28[a]
Diltiazem 10^{-6} M	1.04±0.02	1.00±0.04	4.08±0.58	2.73±0.42
Diltiazem 10^{-5} M	1.01±0.02	1.00±0.04	4.43±0.76	2.94±0.23
Amiloride 10^{-4} M	1.06±0.02	1.16±0.04	4.99±0.48	3.54±0.38
Amiloride 10^{-3} M	1.04±0.02	1.07±0.03	3.97±0.51	3.03±0.31
Developed tension (g)				
NT	3.82±0.40	3.33±0.40	0	0.19±0.07
MCC-135 10^{-9} M	3.83±0.41	3.53±0.55	0	0.36±0.07
MCC-135 10^{-8} M	3.43±0.25	3.27±0.34	0	0.60±0.13
MCC-135 10^{-7} M	3.48±0.43	3.29±0.31	0	0.81±0.11[b]
MCC-135 10^{-6} M	3.34±0.34	2.65±0.26	0	1.24±0.22[c]
Diltiazem 10^{-6} M	2.86±0.37	2.33±0.35	0	0.27±0.07
Diltiazem 10^{-5} M	4.48±0.41	3.56±0.21	0	0.35±0.12
Amiloride 10^{-4} M	3.12±0.31	2.67±0.35	0	0.27±0.08
Amiloride 10^{-3} M	3.41±0.29	3.03±0.38	0	0.54±0.10
Heart rate (beats/min)				
NT	309±22	382±11	0	172±41
MCC-135 10^{-9} M	334±12	380±12	0	233±46
MCC-135 10^{-8} M	329±17	398±10	0	286±16
MCC-135 10^{-7} M	322±15	370±11	0	273±17
MCC-135 10^{-6} M	302±4	357±9	0	287±12
Diltiazem 10^{-6} M	350±17	412±12	0	177±27
Diltiazem 10^{-5} M	308±12	357±6	0	174±43
Amiloride 10^{-4} M	361±13	408±19	0	212±46
Amiloride 10^{-3} M	347±13	415±7	0	166±25

The hearts were subjected to pretreatment with monensin (5 μM, 10 min), zero-flow ischemia (15 min) and reperfusion (30 min). Values are means±S.E.M. for 8 separate hearts. I/R, ischemia and reperfusion.
[a] $P<0.05$ vs. sham.
[b] $P<0.01$ vs. sham.
[c] $P<0.001$ vs. sham.

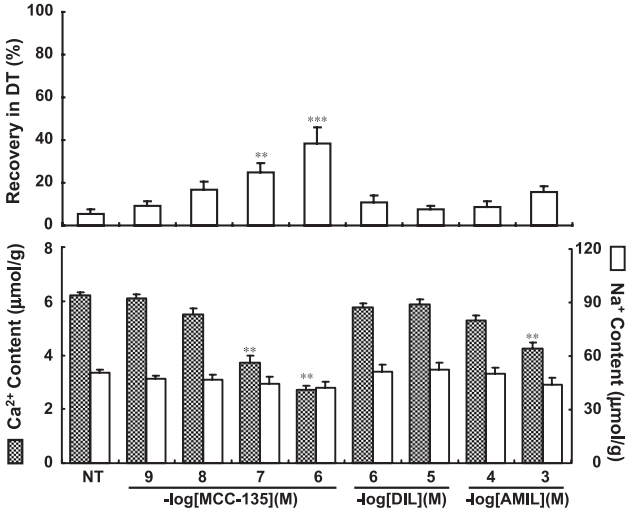

Fig. 5. Effects of MCC-135, diltiazem (DIL) and amiloride (AMIL) on recovery of developed tension, expressed as a percentage of baseline developed tension, (top panel) and ventricular Ca^{2+} and Na^{+} contents normalized to ventricular dry weight (bottom panel) after 30 min of reperfusion following monensin pretreatment (5 μM, 10 min) and 15 min of zero-flow ischemia (protocol B). Drugs were present only during reperfusion. Values are means±S.E.M. for 8 separate hearts. $^{**}P<0.01$, $^{***}P<0.001$ vs. non-treatment (NT). DT, developed tension.

4. Discussion

4.1. Ischemia and reperfusion

The postischemic heart is characterized by contractile dysfunction. Although the pathogenesis of the postischemic contractile dysfunction has not been definitively established, two major theories supported by most experimental evidence suggest that Ca^{2+} overload (Ca^{2+} hypothesis) and the generation of oxygen-derived free radicals (oxyradical

hypothesis) are responsible for the postischemic contractile dysfunction (Gross et al., 1999). The Ca^{2+} and oxyradical hypotheses are not mutually exclusive and are likely to represent different facets of the same pathophysiological cascade. For example, increased free radical formation could cause cellular Ca^{2+} overload, which would damage the contractile apparatus of the myocytes (Zeitz et al., 2002; Bolli and Marban, 1999). Therefore, inhibition of Ca^{2+} overload could be a potential therapeutic approach for protecting the myocardium against ischemia/reperfusion injury.

Ischemia and reperfusion resulted in contractile dysfunction, which was associated with an increase in myocardial Ca^{2+} content. MCC-135 and amiloride improved contractile dysfunction due to ischemia and reperfusion, which was related to a decreased Ca^{2+} content, whereas diltiazem further decreased cardiac contractility without having a significant effect on the myocardial Ca^{2+} content. The effects of amiloride, an inhibitor of the Na^+/H^+ exchanger, reached statistical significance only at the highest dose of 10^{-3} M. Since amiloride has an inhibitory effect on the Na^+/Ca^{2+} exchanger at 10^{-3} M and higher (Kennedy et al., 1986), inhibition of the Na^+/Ca^{2+} exchanger as well as the Na^+/H^+ exchanger may have contributed to the effects of amiloride. This may be supported by a previous suggestion that although inhibition of the Na^+/H^+ exchanger may be of some benefit during low-flow ischemia, additional effects may be necessary to provide more efficient cardioprotection (Khandoudi et al., 1996). Treatment with diltiazem before and during ischemia improved postischemic contractile dysfunction and reduced Ca^{2+} overload (Watts et al., 1987). In contrast, treatment with diltiazem only after reperfusion failed to improve postischemic recovery of cardiac contractile function (Inagaki et al., 2000; Watts et al., 1980), which is consistent with our results and supports the hypothesis that Ca^{2+} overload upon reperfusion is mediated by Ca^{2+} influx via the Na^+/Ca^{2+} exchanger rather than via the L-type Ca^{2+} channel (Inserte et al., 2002; Tani and Neely, 1989). The augmented contractile dysfunction in diltiazem-treated hearts compared to non-treated hearts may be attributable to the negative inotropic effect of diltiazem due to L-type Ca^{2+} channel block rather than increased ischemic damage (Noguchi et al., 1998).

The present results suggest that inhibition of Ca^{2+} overload, at least in part, contributes to the cardioprotective effect of MCC-135 in myocardial ischemia and reperfusion. However, the results cannot rule out the possibility that the cardioprotection provided by MCC-135 is attributable to other mechanisms, such as inhibition of oxidative stress and improvement of energy metabolism, which deserve further investigation. We previously reported that MCC-135 enhances the Ca^{2+} uptake function of the sarcoplasmic reticulum in the post-ischemic/reperfused myocardium (Kawasumi et al., 1999) and the failing myocardium due to cardiomyopathy (Satoh et al., 2001; Satoh and Kitada, 2003), which might have contributed to the improved cardiac function observed in the present study.

4.2. Ischemia and reperfusion with monensin pretreatment

The effects of the drugs on contractile function and ion content were investigated in another protocol in which no-flow ischemia was combined with pretreatment with monensin. Pretreatment with monensin, a Na^+ ionophore, increases the intracellular Na^+ content (van den et al., 1995). No-flow ischemia also increases the intracellular Na^+ content by reducing Na^+/K^+-ATPase activity via a decrease in cardiac ATP level (Wolff et al., 2002) and by activating the Na^+/H^+ exchanger via intracellular acidosis (Avkiran, 1999). Accordingly, no-flow ischemia, when combined with pretreatment with monensin, could effectively cause intracellular Na^+ overload. In fact, myocardial Na^+ overload was observed after 15 min of ischemia following pretreatment with 5 μM monensin for 10 min (Tani and Neely, 1989), which is the same protocol as ours.

In our study, the ventricular Ca^{2+} content of the ischemic/reperfused hearts without pretreatment with monensin was not different from that of sham hearts, suggesting that no-flow ischemia for 15 min followed by reperfusion was not sufficient to cause Ca^{2+} overload. In contrast, the ventricular Ca^{2+} content of the ischemic/reperfused hearts pretreated with monensin was significantly higher than that of the sham hearts. Therefore, the increase in Ca^{2+} content accompanied by cardiac contractile dysfunction in ischemic/reperfused hearts pretreated with monensin is likely to be associated with the intracellular Na^+ overload induced by monensin and the subsequent Ca^{2+} overload mediated by the Na^+/Ca^{2+} exchanger. This hypothesis is supported by the observation that amiloride inhibited the Ca^{2+} overload in the same protocol only at 10^{-3} M, a dose with an inhibitory effect on Na^+/Ca^{2+} exchanger activity, but not at a lower dose (10^{-4} M) with a maximal inhibitory effect on Na^+/H^+ exchanger activity (Kennedy et al., 1986).

Ca^{2+} influx through Ca^{2+} channels could also contribute to the myocardial Ca^{2+} overload. However, the failure of diltiazem to inhibit the Ca^{2+} overload suggests that Ca^{2+} influx through Ca^{2+} channels is not responsible for the Ca^{2+} overload observed under the present experimental conditions. Taken together, these data suggest that the prevention of myocardial Ca^{2+} overload by MCC-135 primarily resulted from an inhibition of Na^+-dependent Ca^{2+} entry, most likely due to actions on the Na^+/Ca^{2+} exchanger.

Inhibition of Na^+-dependent Ca^{2+} influx could theoretically maintain Na^+ overload in return for inhibition of Ca^{2+} overload. However, the inhibition of Ca^{2+} overload with MCC-135 and amiloride was accompanied by decrease in Na^+ content. A similar reduction in Na^+ overload has been reported in anoxia and reoxygenation by 2-[2-[4-(4-nitrobenzeyloxy)phenyl]ethyl]isothiourea (KB-R7943), an inhibitor of the reverse mode of the Na^+/Ca^{2+} exchanger (Ladilov

et al., 1999), though the mechanism underlying this inconsistency remains unclear at present.

4.3. Potential therapeutic efficacy of MCC-135 in ischemic heart diseases

This study demonstrates that MCC-135 improves post-ischemic cardiac contractile dysfunction, which is associated with inhibition of myocardial Ca^{2+} overload. The mechanism of inhibition of cardiac Ca^{2+} overload may include inhibition of intracellular Na^+-dependent Ca^{2+} influx, most likely via the Na^+/Ca^{2+} exchanger.

The results of this study suggest that MCC-135 could ameliorate myocardial injury induced by ischemia and reperfusion in ischemic heart diseases, including acute myocardial infarction. Since drugs can be administered only during revascularization therapy, drugs are required to exert a cardioprotective effect at the time of reperfusion, not before ischemia, in a clinical setting. MCC-135 met this requirement, as the cardioprotective effects of MCC-135 were observed when the drug was present only in the reperfusate. Thus, combination of revascularization by percutaneous coronary intervention or thrombolytic therapy and treatment with MCC-135 could minimize myocardial injury, resulting in maintained cardiac function and reduced mortality.

In fact, MCC-135 reduces levels of cardiac markers, including creatine kinase and troponin T, as well as left ventricular end-diastolic and end-systolic volumes and increases left ventricular ejection fraction in patients with myocardial infarction undergoing percutaneous coronary interventions (Late Breaking Clinical Trials in Scientific Session 2004, American College of Cardiology, by Tzivoni et al.).

4.4. Limitations of the study

We should be cautious about extrapolating the results of this study with isolated rat hearts with global ischemia and reperfusion to clinical settings for some reasons. First, the experimental environment of isolated buffer-perfused hearts is different from that in intact hearts with neuronal control and blood components such as leukocytes. Secondly, myocardial injury induced by global ischemia is likely to be more severe than that induced by regional ischemia (Nighswander-Rempel et al., 2002). Thirdly, it is well established that responses to ischemia are different between smaller species with a faster heart rate, e.g. rats, and larger species with a slower heart rate, e.g. pigs and humans (Rouslin, 1987). Therefore, the effects of MCC-135 may be different, quantitatively and qualitatively, in isolated rat hearts with global ischemia and in intact human hearts with regional ischemia.

In this study, myocardial Ca^{2+} and Na^+ contents were measured by atomic absorption analysis after coronary artery flushing to minimize the contribution of extracellular Ca^{2+} and Na^+. This method, however, does not enable us to strictly discriminate intracellular and extracellular Ca^{2+} and Na^+. Thus, experiments with methods to measure intracellular Ca^{2+} level directly using $^{45}Ca^{2+}$, intracellular Ca^{2+} indicators or electrophysiological techniques should be performed, which would enhance our understanding of the action of MCC-135 on myocardial Ca^{2+} handling and the Na^+/Ca^{2+} exchanger. Moreover, comparison with cardioprotective drugs with completely different effects on intracellular ion levels would provide additional information on the mechanism of action of MCC-135.

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