MCC-134, a Single Pharmacophore, Opens Surface ATP–Sensitive Potassium Channels, Blocks Mitochondrial ATP–Sensitive Potassium Channels, and Suppresses Preconditioning

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Background—MCC-134 (1-[4-(H-imidazol-1-yl)benzoyl]-N-methylcyclobutane-carbothioamide), a newly developed analog of aprikalim, opens surface smooth muscle–type ATP-sensitive potassium (K_ATP) channels but inhibits pancreatic K_ATP channels. However, the effects of MCC-134 on cardiac surface K_ATP channels and mitochondrial K_ATP (mitoK_ATP) channels are unknown. A mixed agonist/blocker with differential effects on the two channel types would help to clarify the role of K_ATP channels in cardioprotection.

Methods and Results—To index mitoK_ATP channels, we measured mitochondrial flavoprotein fluorescence in rabbit ventricular myocytes. MCC-134 alone had little effect on basal flavoprotein fluorescence. However, MCC-134 inhibited diazoxide-induced flavoprotein oxidation in a dose-dependent manner (EC50 = 27 μmol/L). When ATP was included in the pipette solution, MCC-134 slowly activated surface K_ATP currents with some delay (>10 minutes). These results indicate that MCC-134 is a mitoK_ATP channel inhibitor and a surface K_ATP channel opener in native cardiac cells. In cell-pelleting ischemia assays, coapplication of MCC-134 with diazoxide abolished the cardioprotective effect of diazoxide, whereas MCC-134 alone did not alter cell death. These results were reproducible in both rabbit and mouse myocytes. MCC-134 also attenuated the effect of ischemic preconditioning against myocardial infarction in mice, consistent with the results of cell-pelleting ischemia assays.

Conclusions—A single drug, MCC-134, opens surface K_ATP channels but blocks mitoK_ATP channels; the fact that this drug inhibits preconditioning reaffirms the primacy of mitoK_ATP rather than surface K_ATP channels in the mechanism of cardioprotection. (Circulation. 2003;107:1183-1188.)

Key Words: ischemia ■ potassium ■ myocardial infarction

ATP-sensitive K+ channels (K_ATP channels) modulate various physiological and pathophysiological pathways in excitable tissues, including insulin secretion in pancreatic β-cells, vasodilation in smooth muscle cells, and ischemic preconditioning (IPC) in cardiac myocytes.1,2 Pharmacological studies have clearly implicated K_ATP channels in the mechanism of IPC,3 but the identity and subcellular localization of the relevant channels remain uncertain. Cardiac myocytes contain K_ATP channels in both the surface membrane4 and in mitochondria (mitoK_ATP channels).5-7 Selective pharmacological blockers and agonists have implicated mitoK_ATP channels rather than surface K_ATP channels in IPC5,8; however, a recent study in knockout mice suggests that surface K_ATP channels are primary.9

The present controversy could be productively addressed by the use of a single pharmacological agent with directionally opposite effects on mitoK_ATP channels and surface K_ATP channels. Shindo et al10 found that MCC-134, a novel vasorelaxing agent, activates cardiac and smooth muscle–type K_ATP channels but inhibits pancreatic-type K_ATP channels. These unique properties of MCC-134 motivated us to characterize the effect of MCC-134 on mitoK_ATP channels. We found that MCC-134 is an inhibitor of mitoK_ATP channels but an opener of surface K_ATP channels in native cardiac myocytes. Results from cell protection assays and in vivo IPC mouse studies support the concept that mitoK_ATP channels are the key players in cardioprotection.
Methods

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Materials

Collagenase (type II) was purchased from Worthington. MCC-134 was provided by Mitsubishi Pharma Corporation. Diazoxide and DNP were obtained from Sigma Chemical Co. Either diazoxide or MCC-134 was dissolved in DMSO to make stock solution at 100 mM, before being added into experimental solutions. The final concentration of DMSO was <0.3%.

Cell Isolation

New Zealand White rabbits of either sex (1 to 2 kg; Robinson Inc, Clemmons, NC) were anesthetized by intravenous injection of pentobarbitone (30 mg/kg). After confirming the absence of a corneal reflex, hearts were rapidly removed and mounted on a Langendorff apparatus. Ventricular myocytes were isolated by conventional enzymatic dissociation as described previously.7,8,11 Briefly, hearts were perfused with constant flow (12 to 14 mL/min, 37°C) for 5 minutes with normal modified Tyrode solution containing (in mM/L) NaCl 140, KCl 5, CaCl 1, MgCl 1, HEPES 10, and glucose 10 (pH 7.4 with NaOH). 5 minutes of Ca2+-free Tyrode solution, 20 minutes of Ca2+-free Tyrode-containing collagenase (1 mg/mL), and 5 minutes of Ca2+-free Tyrode sequentially. Cells were then cultured on laminin-coated coverslips in M-199 culture medium with 2% fetal bovine serum at 37°C.

Flavoprotein Fluorescence Measurements

Endogenous flavoprotein fluorescence was excited with a xenon arc lamp with a band-pass filter centered at 480 nm, but only during 100 ms of each cycle to minimize photobleaching. Emitted fluorescence was recorded at 530 nm by a photomultiplier tube and digitized.7,11,13 By focusing on individual myocytes with a ×40 objective, fluorescence was monitored from one cell at a time. In some experiments, flavoprotein fluorescence was measured with confocal imaging as described previously.7,11 Briefly, fluorescence was excited by the 488-nm line of an argon laser and emission was recorded at 530 nm by a photomultiplier tube and digitized.7,11

Electrophysiology

For whole-cell patch recordings, the internal pipette solution contained (in mM/L) K-glutamate 120, KCl 25, MgCl 1, EGTA 10, HEPES 10, Mg-ATP 1 (pH 7.2 with KOH). The composition of the external solution is the same as the Tyrode solution used for cell isolation. Currents were elicited every 6 seconds from holding potential of −80 mV by consecutive steps to −40 mV for 100 ms and then to 0 mV for 380 ms. To quantify Icat, currents were measured 200 ms into the second pulse.

Electrophysiology

Myocardial Infarction Studies in Mice

The studies were performed in male ICR (Institute of Cancer Research) mice (weight, 35.2 ± 0.7 g; age, 9.2 ± 0.2 weeks). All mice were maintained in microisolator cages under specific pathogen-free conditions in a room with a temperature of 24°C, 55% to 65% relative humidity, and a 12-hour light-dark cycle.

The experimental preparation has been described in detail.15,16 Briefly, mice were anesthetized with sodium pentobarbitone (60 mg/kg IP) and ventilated by carefully selected parameters.15,16 After administration of antibiotics, the chest was opened through a midline sternotomy, and a nontraumatic balloon occluder was implanted around the mid-left anterior descending coronary artery with an 8–0 nylon suture. To prevent hypotension, blood from a donor mouse was transfused during surgery. Rectal temperature was carefully maintained between 36.7°C and 37.3°C throughout the experiment.

In all groups, myocardial infarction was produced by a 30-minute coronary occlusion followed by 24 hours of reperfusion.15,16 IPC was elicited with a sequence of 6 cycles of 4-minute coronary occlusion and 4-minute reperfusion. Mice were assigned to 6 groups. MCC-134 or vehicle (5% DMSO in saline) was administered in the presence or absence of IPC. Group 1 (control group) underwent the 30-minute occlusion with no prior IPC and no intervention. Mice in group 2 (IPC sham group) were administered 30 minutes before the occlusion/reperfusion cycles, 30 minutes before the 30-minute coronary occlusion, and 10 minutes before reperfusion. In group 5 (MCC + sham IPC group), mice underwent sham IPC and 3 doses of MCC-134 (100 μg/kg IP ×3) were administered at times corresponding to those in group 4. Group 6 was subjected to the same protocol as group 4 except that the mice were given vehicle (5% DMSO solution [5 μL/g IP]) instead of MCC-134. MCC-134 was dissolved in 5% DMSO in normal saline. The final concentration of MCC-134 was 20 μg/mL (volume 5 μL/1 g).

At the conclusion of the study, the occluded/reperfused vascular bed and the infarct were identified by postmortem perfusion of the heart with triphenyltetrazolium chloride and phtalal blue dye.15,16 Infarct size was calculated by computerized videomicroscopy.15,16

Data Analysis

All quantitative data are presented as mean±SEM, and the number of cells or experiments is shown as n. Statistical analysis was performed by means of 1-way ANOVA with Fisher’s least significant difference as the post hoc test. A level of P<0.05 was accepted as statistically significant. In the in vivo myocardial infarction studies, measurements were analyzed by means of a 1-way ANOVA,
followed by unpaired Student's t tests with the Bonferroni correction. The correlation between infarct size and risk region size was assessed by least-squares linear regression analysis. The relation between infarct size and risk region size was compared among groups by means of ANCOVA, with size of the risk region as the covariate.

Results

To examine whether MCC-134 activates mitoKATP channels, mitochondrial flavoprotein fluorescence was measured during exposure to MCC-134. Figure 1A shows that exposure to 100 μmol/L diazoxide reversibly oxidized mitochondrial flavoprotein fluorescence, indicating the opening of mitoKATP channels; in contrast, subsequent exposure to 100 μmol/L MCC-134 had little effect on flavoprotein fluorescence. Summarized data in Figure 1B indicate that diazoxide significantly increased flavoprotein oxidation but that MCC-134 did not, suggesting that MCC-134 is not an opener of mitoKATP channels in rabbit ventricular cells.

We next looked for an inhibitory effect of MCC-134 on mitoKATP channels, as the drug is known to inhibit pancreatic-type KATP channels. As shown in Figure 2A, a first exposure to 100 μmol/L diazoxide alone reversibly increased flavoprotein fluorescence; however, in the presence of MCC-134, repeat exposure to diazoxide did not increase flavoprotein fluorescence. Figure 2B summarizes the pooled data. We previously established that repeated exposures to diazoxide induce comparable degrees of flavoprotein oxidation. Therefore, these results indicate that diazoxide-induced oxidation is suppressed by MCC-134. To examine whether MCC-134 can block already-open mitoKATP channels, we measured flavoprotein fluorescence when MCC-134 was applied after the diazoxide-induced oxidation had reached steady state. Figure 2C shows that MCC-134 reversed the diazoxide-induced oxidation, indicating that MCC-134 has inhibitory action on the open state of mitoKATP channels as well as on the closed state.

To study the concentration dependence of the inhibitory effect of MCC-134 on mitoKATP channels, we measured flavoprotein fluorescence in populations of myocytes by using confocal imaging. Figure 3A indicates that diazoxide-induced mitochondrial oxidation was inhibited by MCC-134, with progressively greater block at increasing concentrations (3 μmol/L; 17.4 ± 1.7%, 10 μmol/L; 23.0 ± 2.0%, 30 μmol/L; 49.9 ± 2.9%, 100 μmol/L; 93.3 ± 2.1%, n = 64 cells). Figure 3B shows the dose-response relation, revealing an EC50 of 27 μmol/L; this value is close to that of the inhibitory action of MCC-134 on pancreatic KATP channels expressed in HEK293T cells.

Next, to test the effect of MCC-134 on native cardiac KATP channels, whole-cell membrane current was recorded with the use of a patch clamp. Figure 4A shows that when 1 mmol/L ATP was included in the pipette solution, exposure to 100 μmol/L MCC-134 had little immediate effect on IK,ATP, but IK,ATP was activated with some delay (≥10 minutes, n = 4 cells). We have recently reported a similar phenomenon with another opener, pinacidil, which is known to shift the sensitivity of KATP channels to ATP, resulting in the opening of KATP channels at higher intracellular ATP levels. To test whether MCC-134 also shifts the sensitivity of surface KATP channels to intracellular ATP, IK,ATP was recorded during rapid intracellular ATP depletion by dinitrophenol (DNP) in the continued presence of MCC-134. At the chosen concentration, DNP alone does not suffice to open surface KATP channels, but the ATP depletion potentiates the action of pharmacological openers. As shown in Figure 3B, 7 minutes of exposure to MCC-134 alone did not activate KATP channels; however, exposure to DNP in the continued presence of MCC-134 induced rapid activation of surface KATP channels. Note that this activation reversed rapidly on washout of DNP. Taken together, these results indicate that...
MCC-134 is an activator of surface K_ATP channels but an inhibitor of mitoK_ATP channels in ventricular cells.

These unique properties of MCC-134 motivated us to determine which effect is dominant in cardioprotection. If surface channels are important, MCC-134 alone should be cardioprotective; if mitochondrial channels are key, MCC-134 should block cardioprotection. To test this, a cell-pelleting model was used, and cell death was quantified by confocal microscopy (see Methods section). Figure 5 shows summarized data from 5 rabbits, indicating that diazoxide has a significant cardioprotective effect (diazoxide: 25.1 ± 4.5% trypan blue staining after 60 minutes [mean ± SEM] versus control: 55.0 ± 6.4%, P < 0.01). MCC-134 alone has no significant effect on cell death (MCC-134: 41.6 ± 4.9%, versus control, NS); however, MCC-134 fully abolished the cardioprotective effect of diazoxide (41.6 ± 5.6%, P < 0.05 versus diazoxide, NS versus control).

These conclusions contrast with those recently reached on the basis of studies of Kir6.2 knockout mice, which appeared to indicate that surface K_ATP channels figure prominently in cardioprotection in the mouse. To exclude possible species-specific effects, we performed cardioprotection assays with mice. Figure 6 shows summarized data from 5 cell-pelleting experiments (15 mice). Consistent with the cell-pelleting experiments with rabbit cardiomyocytes, diazoxide has a significant cardioprotective effect (diazoxide: 24.1 ± 5.7% trypan blue staining after 60 minutes [mean ± SEM] versus control: 59.7 ± 5.4%, P < 0.01). MCC-134 alone has no significant effect on cell death (MCC-134: 55.6 ± 4.6% versus control, NS); however, MCC-134 fully abolished the cardioprotective effect of diazoxide (62.8 ± 6.2%, P < 0.05 versus diazoxide, NS versus control).

Figure 3. Concentration-dependent inhibitory effect of MCC on diazoxide-induced oxidation. A, Time course of mean fluorescence level for 64 individual cells induced by diazoxide and MCC-134. Note that additional application of MCC inhibited diazoxide-induced flavoprotein oxidation. B, Concentration-response relations between MCC-134 and flavoprotein oxidation.

Figure 4. Effect of MCC-134 on surface K_ATP channels. Time course of \( I_{\text{K_ATP}} \) at 0 mV induced by 100 \( \mu \text{mol/L} \) MCC-134 alone. B, Rapid activation of \( I_{\text{K_ATP}} \) by exposure to 100 \( \mu \text{mol/L} \) DNP in the continued presence of 100 \( \mu \text{mol/L} \) MCC-134. Summarized data for \( I_{\text{K_ATP}} \) measured 5 minutes after exposure to MCC alone or just after application of DNP in the continued presence of MCC.

Figure 5. Cell-pelleting model of ischemic injury in rabbit hearts. Columns indicate percent cell death induced by 60 minutes of ischemia; error bars indicate SEM. Data are from 5 rabbits. \(* P < 0.05, ** P < 0.01, \) respectively.
Next, we performed in vivo myocardial infarction studies. A total of 145 mice were used in this investigation. We used 70 mice as blood donors and another 5 mice for hemodynamic measurements. Protocol was completed in 71 mice (12 for the pilot studies, and 59 for the formal studies).

In the pilot studies, we found that 50 to 100 μg/kg of MCC-134 did not cause any changes in arterial pressure and heart rate in non preconditioned mice. A single dose of MCC-134 (50 μg/kg [n=3] or 100 μg/kg [n=4]) administered 30 minutes before the 6 occlusion/reperfusion cycles could not totally block the IPC effect on infarct size. Thus, we used a protocol in which we administered three doses of MCC-134 (100 μg/kg IP ×3) 30 minutes before the 6 occlusion/reperfusion cycles, 30 minutes before the 30-minute coronary occlusion, and 10 minutes before reperfusion.

There were no significant differences among the 6 groups with respect to left ventricular weight or weight of the region at risk (data not shown). In group 1 (n=10), infarct size averaged 49.8±2.7% of the region at risk (Figure 7). Similar results were obtained in groups 2 (n=10, 49.3±1.8%) and 5 (n=9, 49.0±2.7%) (Figure 7), indicating that MCC-134 in itself had no effect on infarct size (Figure 7). However, a sequence of 6 cycles of 4-minute occlusion and 4-minute reperfusion ending 10 minutes before the 30-minute occlusion (group 3) markedly reduced infarct size to 13.1±2.0% of the region at risk, indicating a powerful IPC effect against infarction. This cardioprotective effect was significantly inhibited by MCC-134 (group 4; 34.5±3.7% of the risk region; P<0.05 versus group 3) (Figure 7). Administration of vehicle (group 6) had no effect on infarct size (16.4±2.5% of the region at risk) (Figure 7).

Discussion

The present study demonstrated that MCC-134 inhibits mitoKATP channels but activates surface KATP channels in native cardiac myocytes. These results parallel previous observations that MCC-134 activates KATP channels composed of Kir6.2+SUR2A (the cardiac surface isoform) but inhibits Kir6.2+SUR1 (pancreatic) channels, indicating the similarity between mitoKATP channels and pancreatic KATP channels. Considering that the drug sensitivity of KATP channels depends on the SUR subtype, SUR1 might logically be thought to form part of mitoKATP channels. Grover and Garlid reported that they could detect an SUR-like protein in a mitochondrial compartment, but this protein is much smaller than SUR1. More work is needed to establish the precise molecular identity of mitoKATP channels.

The identification of a single agent that can simultaneously open surface KATP channels and block mitoKATP channels enables a simple test of the roles of the two channel types. The observation that MCC-134 blocks cardioprotection both in vivo and in vitro convincingly argues for the primacy of mitoKATP channels in the mechanism of IPC.

Our data from mice contrast with those recently reached on the basis of studies of Kir6.2 knockout mice. Those animals lack surface KATP channels but have intact flavoprotein fluorescence responses to diazoxide, hinting that mitoKATP channels are present. Kir6.2 knockout mice do not manifest IPC. Infarct size is not decreased by prior episodes of conditioning ischemia in contrast to wild-type mice. The interpretation of such data at face value hinges on the presumption that ischemic injury is no worse in the knockouts. Such appears not to be the case. During ischemia, hearts from Kir6.2 knockouts had contracture more intensely and more rapidly than control hearts; afterward, functional recovery was much worse in the Kir6.2 knockouts, in the absence of any preconditioning stimulus. Thus, knockout of surface KATP channels might artificially enhance ischemic injury and cancel the effect of IPC, undermining the conclusions reached by Suzuki et al.

Shindo et al suggested that MCC-134 is probably beneficial for treating patients with diabetes mellitus accompanied with hypertension because of its vasodilatory action and acceleration of insulin secretion. From a cardiac viewpoint, MCC-134 blunts cardioprotection and opens surface KATP channels; the latter may favor ischemic arrhythmias. In fact, from a strictly cardiac viewpoint, the perfect modulator of KATP channels would have effects opposite to those of MCC-134: A simultaneous opener of mitoKATP channels and...
blocker of surface $K_{ATP}$ channels would be expected to mitigate ischemic injury while blunting arrhythmias. The fact that MCC-134 has the opposite profile should not overshadow the conceptual importance of the demonstration that one single drug can have directionally opposite effects on two key $K_{ATP}$ channel subtypes.

**Acknowledgments**

This work was supported by National Institutes of Health grants (R37 HL36957 to Dr Marbán; HL43151, R37 HL55757, and HL68088 to Dr Bolli; and ROI HL52598 to Dr O’Rourke), an American Heart Association Ohio Valley Affiliate (G020634 and HL68088 to Dr Bolli; and ROI HL52598 to Dr O’Rourke), and a Banyu Fellowship in Cardiovascular Medicine (to Dr Akao). Dr Marbán holds the Michel Mirowski, MD, 0265087B to Dr Guo), and a Banyu Fellowship in Cardiovascular Medicine (to Dr Akao). Dr Marbán holds the Michel Mirowski, MD, 0265087B to Dr Guo), and a Banyu Fellowship in Cardiovascular Medicine (to Dr Akao). Dr Marbán holds the Michel Mirowski, MD, 0265087B to Dr Guo).

**References**

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Circulation. 2003;107:1183-1188; originally published online February 10, 2003; doi: 10.1161/01.CIR.0000051457.64240.63
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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