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

Norihito Sasaki, MD, PhD[*], **Mitsushige Murata, MD, PhD**[*], **Yiru Guo, MD**, **Su-Hyun Jo, PhD**, **Andreas Ohler, MD**, **Masaharu Akao, MD, PhD**, **Brian O'Rourke, PhD**, **Rui-Ping Xiao, MD, PhD**, **Roberto Bolli, MD**, and **Eduardo Marbán, MD, PhD**
Laboratory of the Institute of Molecular Cardiobiology (N.S., M.M., A.O., M.A., B.O., E.M.), Johns Hopkins University, Baltimore, Md; Cardiovascular Sciences (S.-H.J., R.-P.X.), Gerontology Research Center, National Institute on Aging, National Institute of Health, Baltimore, Md; and the Experimental Research Laboratory (Y.G., R.B.), Division of Cardiology, University of Louisville and the Jewish Heart and Lung Institute, Louisville, Ky

Abstract

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 (EC_{50}=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.

Keywords

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-

Correspondence: to Eduardo Marbán, MD, PhD, Institute of Molecular Cardiobiology, Johns Hopkins University, 720 Rutland Ave/ Ross 844, Baltimore, MD 21205. marban@jhmi.edu.
[*]Drs Sasaki and Murata contributed equally to this work.

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 membrane[4] 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 IPC[5,7,8]; 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 al[10] 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 mmol/L 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 mmol/L) NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10, and glucose 10 (pH 7.4 with NaOH), 5 minutes of Ca^{2+}-free Tyrode solution, 20 minutes of Ca^{2+}-free Tyrode-containing collagenase (1 mg/mL), and 5 minutes of Ca^{2+}-free Tyrode sequentially. Cells were then cultured on laminin-coated coverslips in M-199 culture medium with 2% fetal bovine serum at 37°C.

Single cardiac myocytes were isolated from the hearts of 2- to 3-month-old mice by means of an enzymatic technique, as described previously.[12] Experiments were performed 1 to 2 hours after isolation.

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.[7,8,11,13] 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 collected at 520±15-nm. At the beginning of each experiment, 50 to 70 cells per field were selected, and the time course of flavoprotein fluorescence changes in the various selected individual cells was monitored simultaneously. At the end of each experiment, the redox potentials in each cell were normalized to the corresponding fully reduced (CN^-) and fully oxidized (FCCP) fluorescence values. All experiments were performed at room temperature (22° to 23°C).

Electrophysiology

For whole-cell patch recordings, the internal pipette solution contained (in mmol/L) K-glutamate 120, KCl 25, $MgCl_2$ 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 $I_{K,ATP}$, currents were measured 200 ms into the second pulse.

Cell-Pelleting Model: Simulated Ischemia and Cellular Injury

A cell-pelleting model of ischemia modified from Vander Heideet al[14] was used to quantify myocyte injury. In brief, adult rabbit or mouse ventricular cells were washed with incubation buffer (M-199 HEPES-buffered) after cell isolation. Aliquots (0.5 mL) of suspended cells were placed into a microcentrifuge tube and centrifuged for 60 seconds at 1200*g*. Approximately 0.25 mL of excess supernatant was removed to leave a thin fluid layer above the pellet, and 0.2 mL of mineral oil was layered on the top to prevent gaseous diffusion. After 60 minutes, 5 μL of cell pellet was sampled through the oil layer and mixed with 75 μL of 85 mOsm hypotonic staining solution: (in mmol/L) $NaHCO_3$ 11.9, KH_2PO_4 0.4, KCl2.7, $MgSO_4$ 0.8, and $CaCl_2$ 1, with 0.5% glutaraldehyde and 0.5% trypan blue. Cells were imaged by confocal microscopy and those permeable to trypan blue were counted by a blinded observer and expressed as a percentage of the total cells counted after randomly taking at least 3 images (>300 cells) from each sample. The small percentage of cells (≈20%) that were nonviable at the beginning of the experiment were mostly rounded and had been damaged as a consequence of the enzymatic isolation process. The osmotic fragility of cells induced by hypoxia was quantified as percentage of the vital cells (unstained cells) at the beginning of each experiment. In nonpelleted control cells suspended in oxygenated buffer with or without drugs, there was no significant change in the percentage of stained cells after 60 minutes of incubation. Pelleting experiments were performed at 37°C.

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 pentobarbital (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° 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) served as the control for group 3; in these mice, the chest was opened for 1 hour (interval corresponding to the duration of the sequence of six cycles of 4-minute occlusion and 4-minute reperfusion in group 3) before 30 minutes of occlusion followed by 24 hours of reperfusion. Mice in group 3 (IPC group) underwent 6 cycles of 4-minute occlusion and 4-minute reperfusion followed, 10 minutes later, by 30 minutes of coronary occlusion and 24 hours of reperfusion. In group 4 (MCC +IPC group), 3 doses of MCC-134 (100 μg/kg IP ×3) were administered 30 minutes before the 6 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 phthalo blue dye.[15,16] Infarct size was calculated by computerized videoplanimetry.[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 mitoK_{ATP} 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 mitoK_{ATP} 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 mitoK_{ATP} channels in rabbit ventricular cells.

We next looked for an inhibitory effect of MCC-134 on mitoK_{ATP} channels, as the drug is known to inhibit pancreatic-type K_{ATP} 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.[7] Therefore, these results indicate that diazoxide-induced oxidation is suppressed by

MCC-134. To examine whether MCC-134 can block already-open mitoK_{ATP} 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 mitoK_{ATP} channels as well as on the closed state.

To study the concentration dependence of the inhibitory effect of MCC-134 on mitoK_{ATP} 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 EC_{50} of 27 μmol/L; this value is close to that of the inhibitory action of MCC-134 on pancreatic K_{ATP} channels expressed in HEK293T cells.[10]

Next, to test the effect of MCC-134 on native cardiac K_{ATP} 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 $I_{K,ATP}$, but $I_{K,ATP}$ was activated with some delay (>10 minutes, n=4 cells). We have recently reported a similar phenomenon with another opener, pinacidil,[17] which is known to shift the sensitivity of K_{ATP} channels to ATP, resulting in the opening of K_{ATP} channels at higher intracellular ATP levels.[18] To test whether MCC-134 also shifts the sensitivity of surface K_{ATP} channels to intracellular ATP, $I_{K,ATP}$ 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 K_{ATP} channels, but the ATP depletion potentiates the action of pharmacological openers.[19] As shown in Figure 3B, 7 minutes of exposure to MCC-134 alone did not activate K_{ATP} channels; however, exposure to DNP in the continued presence of MCC-134 induced rapid activation of surface K_{ATP} channels. Note that this activation reversed rapidly on wash-out 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 cardio-protective 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.[9] 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).

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 nonpreconditioned mice. A single dose of MCC-134 (50 μg/kg [n=3] or 100 μg/kg [n=4] IP) 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 mitoK_{ATP} channels but activates surface K_{ATP} channels in native cardiac myocytes. These results parallel previous observations that MCC-134 activates expressed K_{ATP} channels composed of Kir6.2+SUR2A (the cardiac surface isoform) but inhibits Kir6.2+SUR1 (pancreatic) channels,[10] indicating the similarity between mitoK_{ATP} channels and pancreatic K_{ATP} channels. Considering that the drug sensitivity of K_{ATP} channels depends on the SUR subtype, SUR1 might logically be thought to form part of mitoK_{ATP} channels. Grover and Garlid[20] reported that they could detect an SUR-like protein from a mitochondrial compartment, but this protein is much smaller than SUR1. More work is needed to establish the precise molecular identity of mitoK_{ATP} channels.

The identification of a single agent that can simultaneously open surface K_{ATP} channels and block mitoK_{ATP} 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 mitoK_{ATP} 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.[9] Those animals lack surface K_{ATP} channels but have intact flavoprotein fluorescence responses to diazoxide, hinting that mitoK_{ATP} channels are present.[9] Kir6.2 knockout mice do not manifest IPC: Infarct size is not decreased by prior episodes of conditioning ischemia,[9] 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.[9] Thus,

knockout of surface K_{ATP} channels might artificially enhance ischemic injury and cancel the effect of IPC, undermining the conclusions reached by Suzuki et al.[9]

Shindo et al[10] 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 cardiocentric viewpoint, MCC-134 blunts cardioprotection and opens surface K_{ATP} channels; the latter may favor ischemic arrhythmias.[19] In fact, from a strictly cardiac viewpoint, the perfect modulator of K_{ATP} channels would have effects opposite to those of MCC-134: A simultaneous opener of mitoK_{ATP} channels and blocker of surface K_{ATP} channels would be expected to mitigate ischemic injury while blunting arrhythmias.[21] 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.

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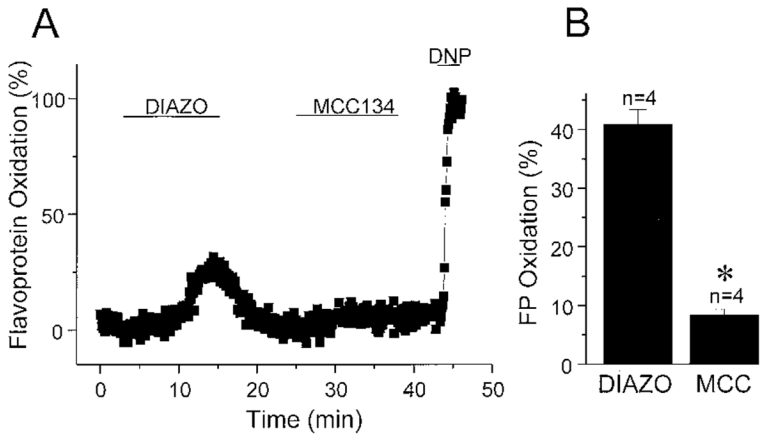

Figure 1.

Effect of MCC-134 on basal flavoprotein fluorescence. A, Time course of flavoprotein fluorescence induced by 100 μmol/L diazoxide and 100 μmol/L MCC-134 in one cell. Flavoprotein fluorescence was measured with photomultiplier tubes. B, Summarized data for diazoxide (DIAZO) and MCC-134 (MCC)-induced flavoprotein oxidation.

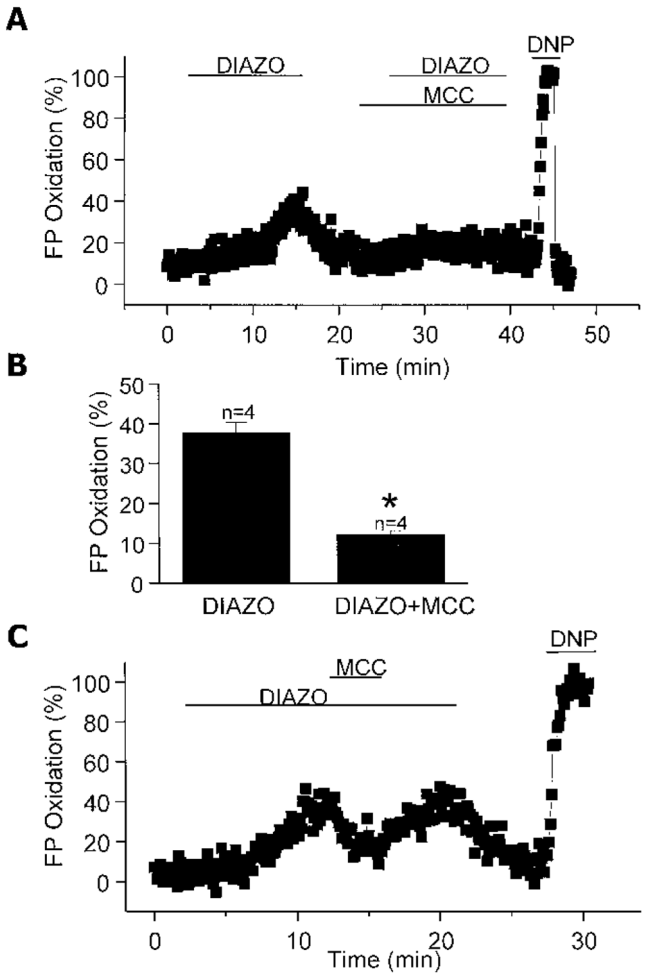

Figure 2.

Inhibitory effect of MCC-134 on diazoxide-induced flavoprotein oxidation. A, In the continued presence of MCC, diazoxide failed to induce flavoprotein oxidation. Flavoprotein fluorescence was measured with photomultiplier tubes. B, Summarized data for diazoxide-induced oxidation in the absence and presence of MCC. C, Additional application of MCC also inhibited diazoxide-induced flavoprotein oxidation.

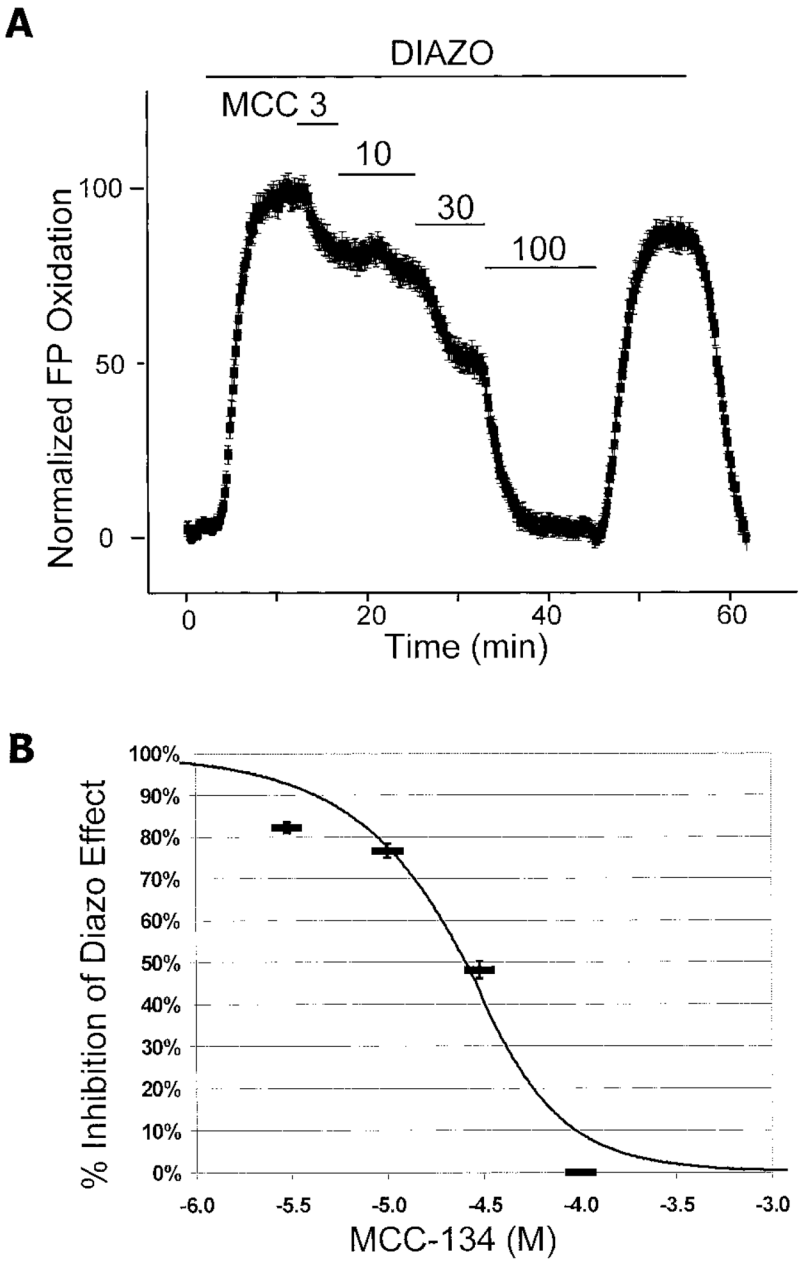

Figure 3.

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.

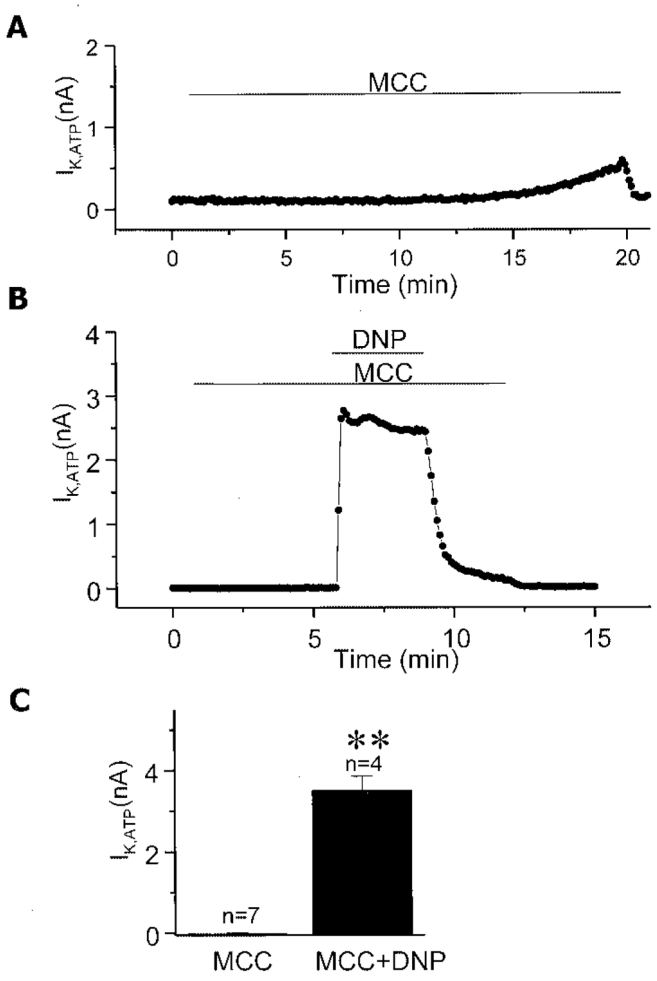

Figure 4.

Effect of MCC-134 on surface K_{ATP} channels. Time course of $I_{K,ATP}$ at 0 mV induced by 100 μmol/L MCC-134 alone. B, Rapid activation of $I_{K,ATP}$ by exposure to 100 μmol/L DNP in the continued presence of 100 μmol/L MCC-134. Summarized data for $I_{K,ATP}$ measured 5 minutes after exposure to MCC alone or just after application of DNP in the continued presence of MCC.

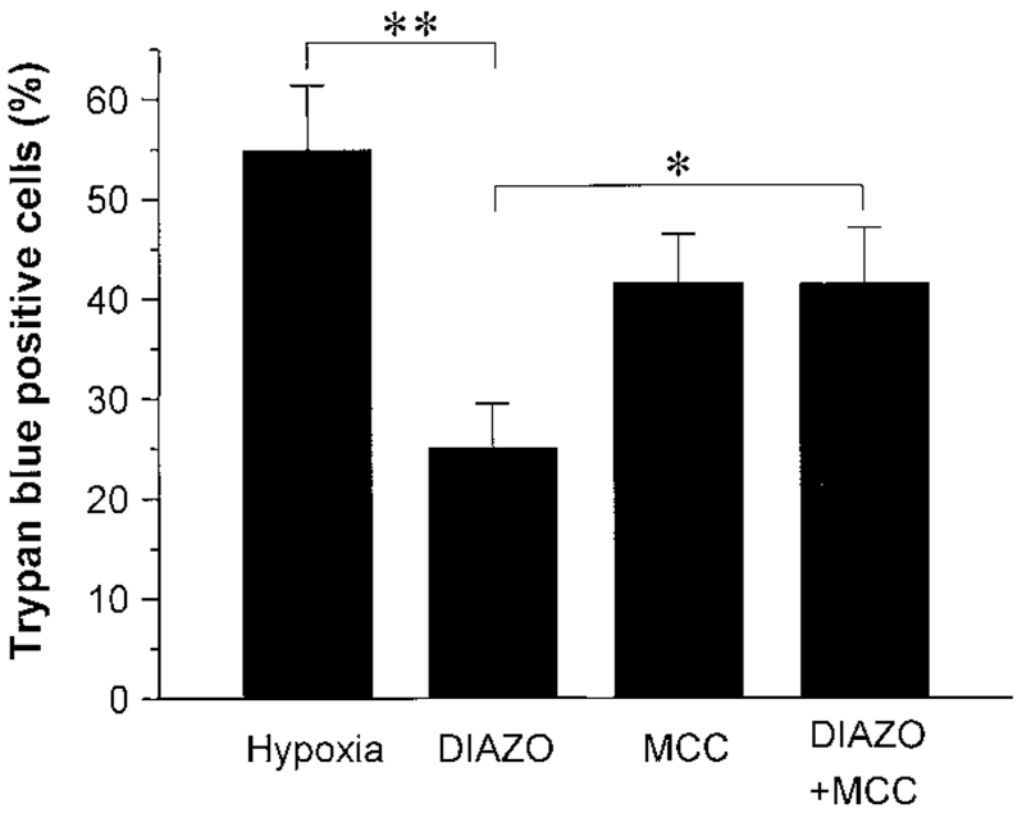

Figure 5.
Cell-pelleting model of ischemic injury in rabbit hearts. Columns indicate percent cell death induced by 60 minutes of ischemia; error bars indicate SEM. Data are from 5 rabbits. **P*<0.05, ***P*<0.01, respectively.

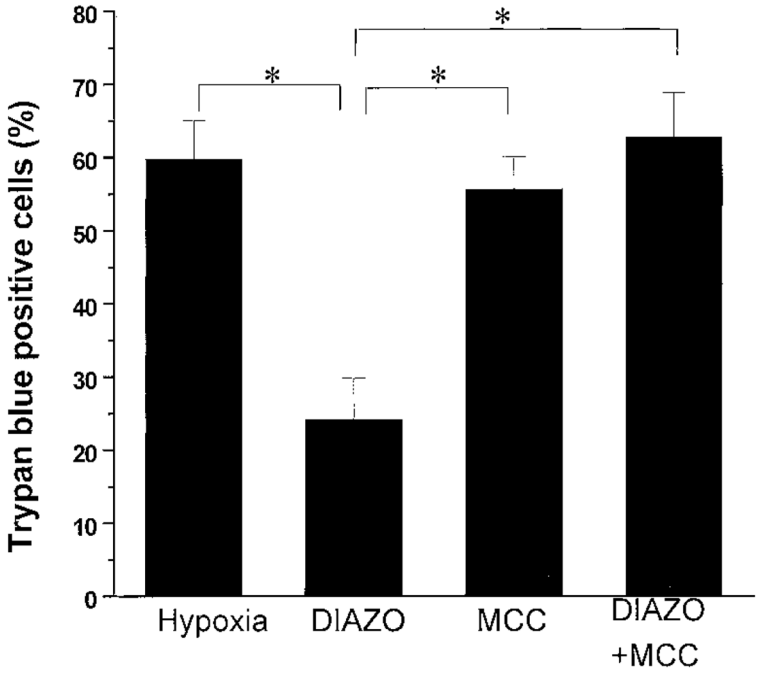

Figure 6.
Cell-pelleting model of ischemic injury in mice hearts. Columns indicate percent cell death induced by 60 minutes of ischemia. Data are from 5 experiments, 15 mice. *$P<0.05$.

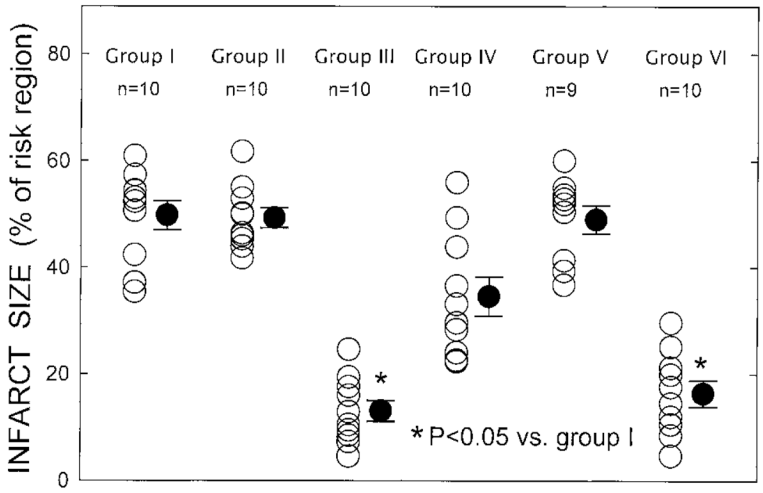

Figure 7.

In vivo myocardial infarct size. Groups 1 (control group), 2 (IPC sham group), 3 (IPC group), 4 (MCC+IPC group), 5 (MCC+sham IPC group), and 6 (vehicle+sham IPC group). ○, individual mice; ●, mean±SEM.