The A3 Adenosine Receptor Agonist CP-532,903 [N6-(2,5-Dichlorobenzyl)-3’-aminoadenosine-5’-N-methylcarboxamide] Protects against Myocardial Ischemia/Reperfusion Injury via the Sarcolemmal ATP-Sensitive Potassium Channel

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ABSTRACT

We examined the cardioprotective profile of the new A3 adenosine receptor (AR) agonist CP-532,903 [N6-(2,5-dichlorobenzyl)-3’-aminoadenosine-5’-N-methylcarboxamide] in an in vivo mouse model of infarction and an isolated heart model of global ischemia/reperfusion injury. In radioligand binding and cAMP accumulation assays using human embryonic kidney 293 cells expressing recombinant mouse ARs, CP-532,903 was found to bind with high affinity to mouse A3ARs (Ki = 9.0 ± 2.5 nM) and with high selectivity versus mouse A1AR (100-fold) and A2AARs (1000-fold). In vivo ischemia/reperfusion experiments, pre-treating mice with 30 or 100 μg/kg CP-532,903 reduced infarct size from 59.2 ± 2.1% of the risk region in vehicle-treated mice to 42.5 ± 2.3 and 39.0 ± 2.9%, respectively. Likewise, treating isolated mouse hearts with CP-532,903 (10, 30, or 100 nM) concentration dependently improved recovery of contractile function after 20 min of global ischemia and 45 min of reperfusion, including developed pressure and maximal rate of contraction/relaxation. In both models of ischemia/reperfusion injury, CP-532,903 provided no benefit in studies using mice with genetic disruption of the A3AR gene, A3 knockout (KO) mice. In isolated heart studies, protection provided by CP-532,903 and ischemic preconditioning induced by three brief ischemia/reperfusion cycles were lost in Kir6.2 KO mice lacking expression of the pore-forming subunit of the sarcolemmal ATP-sensitive potassium (KATP) channel. Whole-cell patch-clamp recordings provided evidence that the A3AR is functionally coupled to the sarcolemmal KATP channel in murine cardiomyocytes. We conclude that CP-532,903 is a highly selective agonist of the mouse A3AR that protects against ischemia/reperfusion injury by activating sarcolemmal KATP channels.

A3 adenosine receptor (AR) agonists have been shown to effectively limit infarct size and reduce contractile dysfunction in several different animal models of ischemia/reperfusion injury (Auchampach et al., 1997b, 2003; Tracey et al., 1997, 1998, 2003; Jordan et al., 1999; Thourani et al., 1999; Ge et al., 2004, 2006). A3AR agonists are attractive as cardioprotective agents because they do not alter systemic hemodynamic parameters in nonrodent species and are effective if administered before the ischemic event or only during reperfusion (Auchampach et al., 1997b, 2003; Tracey et al., 1997, 1998, 2003; Jordan et al., 1999; Thourani et al., 1999; Ge et al., 2004, 2006). The most widely available A3AR agonists that have been tested in experimental animal models of ischemia/reperfusion injury include the N6-benzyladenosine-5’-N-methylcarboxamide derivative IB-MECA and its

2-chloro derivative CI-IB-MECA (Fig. 1). Although IB-MECA and Cl-IB-MECA are potent A3AR agonists, they exhibit moderate selectivity. In radioligand binding assays, the selectivity of IB-MECA and Cl-IB-MECA has been reported to range from 6 to 2500-fold versus the A2AR and 4 to 1400-fold versus the A2AAR, depending on the species and the assay conditions (Gallo-Rodriguez et al., 1994; Kim et al., 1994; Hill et al., 1997; Takano et al., 2001; Murphree et al., 2002).

In the present investigation, we characterized the cardioprotective profile of the A3AR agonist CP-532,903 in an isolated mouse heart model of global ischemia and reperfusion and an in vivo mouse model of infarction. The goal of this work was to examine the cardioprotective effectiveness of CP-532,903 and to confirm whether it mediates cardioprotection via the A3AR. A second goal of this investigation was to determine whether A3AR activation provides ischemic protection by facilitating opening of the sarcolemmal isoform of the ATP-sensitive potassium (KATP) channel. This second question was addressed using Kir6.2 gene knockout (KO) mice lacking the pore-forming subunit of the sarcolemmal KATP channel (Suzuki et al., 2002).

Materials and Methods

Materials

Cell culture reagents, G418 sulfate, and Lipofectamine were purchased from Invitrogen (Carlsbad, CA). CP-532,903 was provided by Pfizer Inc. (Groton, CT), adenosine deaminase and Liberase Blendzyme I were obtained from Roche Applied Biosciences (Indianapolis, IN), E-4031 was purchased from Wako (Osaka, Japan), and all other drugs and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Stably transfected human embryonic kidney (HEK) 293 cells expressing mouse A3Rs and [125I]I-AB-MECA were prepared, as described previously (Auchampach et al., 1997a,b; Kreckler et al., 2006). cAMP and histamine radioimmunoassay kits were obtained from GE Healthcare (Piscataway, NJ) and Immunotech (Marseille, France).

Animals

All experiments were performed with 10 to 14-week-old male mice weighing ~24 to 32 g. Wild-type C57BL/6 mice were purchased from Taconic Farms Inc. (Germantown, NY). A3 KO mice were provided by Dr. Marlene Jacobson (Merck Research Laboratories, West Point, PA). Kir6.2 KO mice were provided by Dr. Susumu Seino (Kobe University, Kobe, Japan) and were generated by Dr. Aaron Fisher (University of Pennsylvania, Philadelphia, PA), as described previously (Miki et al., 1998). All animals used in the study received humane care in accordance with the guidelines established by the Medical College of Wisconsin, which conform to the Institute of Laboratory Animal Resources (1996).

Radioligand Binding Assays

Competition radioligand binding assays were conducted with membranes prepared from HEK 293 cells expressing recombinant mouse A3 or A2ARs using the agonist radioligand [125I]I-AB-MECA (Auchampach et al., 1997a,b; Kreckler et al., 2006). Incubations were conducted in 100 μl of buffer (10 mM Na-HEPES, pH 7.4, 1 mM EDTA, 5 mM MgCl2, 1 U/ml adenosine deaminase) with ~0.3 nM [125I]I-AB-MECA and competitors at room temperature for 3 h, after which bound and free radioligand were separated by filtration over GF/C grade glass fiber filters (Brandel, Gaithersburg, MD). Nonspecific binding was defined by the presence of 100 μM adenosine-5′-N-ethylcarboxamide in the assays. K values for high-affinity agonist binding were calculated as described previously (Auchampach et al., 1997a,b; Kreckler et al., 2006).

cAMP Assays

HEK 293 cells expressing recombinant mouse ARs were detached using phosphate-buffered saline containing 5 mM EDTA and resuspended at 50,000 cells/tube in Dulbecco’s modified Eagle’s medium with 25 mM HEPES, pH 7.4, 1 U/ml adenosine deaminase, and 20 μM Ro 20,1724 to inhibit phosphodiesterases. Cells were incubated with agonists for 15 min at 37°C with shaking. Reactions were terminated by addition of 0.15 N HCl. [cAMP] in the acid extract was determined by radioimmunoassay. MRS 1754 (1 μM) was included in assays with HEK 293 cells expressing A2A or A3ARs to block endogenous A2ARs expressed in HEK 293 cells.

Langendorff-Perfused Mouse Heart Model

Experimental Preparation. Male mice (10–12 weeks of age, 23.7 ± 0.4 g body weight) were anesthetized with sodium pentobarbital (100 mg/kg i.p.). As soon as deep anesthesia was achieved, the hearts were removed and arrested in ice-cold perfusion solution. The hearts were cannulated via the aorta and perfused retrogradely by the Langendorff method at a continuous pressure of 80 mm Hg using Krebs-Henseleit buffer containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl2, 2.5 mM CaCl2, 1.2 mM KH2PO4, 0.5 mM EDTA, 25 mM NaHCO3, and 11 mM glucose. The buffer was equilibrated with 95% O2/5% CO2 at 37°C to maintain the pH at 7.4 and filtered through an in-line Sterivex filter unit (0.22 μm; Millipore, Bedford, MA) to remove particulate matter. For each heart, the left ventricle was drained by inserting a short polyethylene tube through the apex, and a fluid-filled balloon connected to a pressure transducer was inserted into the left ventricle via the mitral valve. The balloon was connected to a pressure transducer (ADInstruments, Colorado Springs, CO) for continuous measurement of left ventricular pressure. The hearts were immersed in perfusion buffer maintained at 37°C, and the balloons were inflated to achieve end-diastolic pressures of 5 to 10 mm Hg. Coronary flows were monitored by an in-line flow probe connected to a flowmeter (model T206; Transonics Systems Inc., Ithaca, NY). The left ventricular pressure signals were acquired continuously using a PowerLab data acquisition system (ADInstruments) and processed (Chart software) to yield heart rates and left ventricular dP/dts.

Global Ischemia/Reperfusion Protocol. Hearts were perfused for 20 min to allow for stabilization and then perfused for an additional 15 min while pacing at 420 beats/min (ventricular pacing with 2-ms square waves at a, voltage of 20% above threshold). Baseline functional measurements were acquired immediately before subjecting the hearts to 20 min of normothermic no-flow ischemia and 45 min of reperfusion achieved by closing and opening an in-line stopcock. To examine the effect of CP-532,903 on functional recovery, hearts were perfused with buffer containing the indicated concentrations of agonists for 10 min before ischemia. In studies of ischemic
preconditioning (IPC), the hearts were subjected to three cycles of 3-min occlusion/2-min reperfusion before the 20-min occlusion.

**In Vivo Mouse Model of Infarction**

**Experimental Preparation.** Male mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.) and prepared for surgery, as described previously (Black et al., 2002; Ge et al., 2006). In brief, the mice were intubated using PE-60 tubing, ventilated (model 845; Hugo Sachs Elektronik, Hugstetten, Germany) at a rate of 100 to 110 beats/min and a tidal volume of 200 to 250 μl using room air supplemented with oxygen, and fitted with electrodes to obtain the electrocardiogram (EKG) using the limb lead II configuration. The heart was exposed via a lateral incision at the level of the fourth intercostal space and a ligature (8-0 nylon suture) was placed around the left coronary artery ~1 to 3 mm from the tip of the left atria. The ligature was used to induce coronary occlusion and reperfusion by gently tightening the snare around a piece of wetted gauze. Approximately 15 min after completing the ischemia/reperfusion protocol, the chest cavity was closed using 6-0 polypropylene suture, and the mice were allowed to recover.

**Ischemia/Reperfusion Protocol.** After a 30-min stabilization period after surgery, all mice were subjected to 30 min of LAD occlusion and 24 h of reperfusion. Successful performance of occlusion and reperfusion was verified by visual inspection (i.e., change in color of the ischemic myocardium) and by changes in the ECG. CP-532,903 or equivalent vehicle was administered as an i.v. bolus 10 min before the coronary artery occlusion. Heart rate was monitored at baseline and during the 30-min occlusion period from the ECG recording.

**Measurement of Ischemic Area and Infarct Size.** After 24 h of reperfusion, infarct size was measured by dual staining with phthaldehyde blue dye and triphenyltetrazolium chloride (Black et al., 2006). To delineate the ischemic area at risk, the LAD was reoccluded in situ while a 5% solution of phthalo blue dye was passed through measurements in the absence of substrates. Chemiluminescence was monitored in a Modulus Luminometer (Turner Biosystems, Sunnyvale, CA) at room temperature for 120 s. The rate of mitochondrial respiration was assessed using a chemiluminescence-based method utilizing firefly luciferase and luciferin (ATP Determination Kit; Invitrogen), as described previously (Ljubkovic et al., 2007). The reaction solution contained respiration buffer containing 130 mM KCl, 5 mM K₂HPO₄, 20 mM MOPS, pH 7.2, 2.5 mM EGTA, 0.001 mM Na₃P₂O₇, and 0.1% bovine serum albumin. State 2 respiration was stimulated with a combination of pyruvate and malate (5 mM each) as substrates. ADP-stimulated state 3 respiration was measured in the presence of 250 μM ADP, and state 4 respiration was measured after added ADP was consumed. The respiratory control ratio was calculated as a ratio of the state 3 rate divided by the state 4 rate.
mitochondrial ATP production was calculated from standard curves generated with defined ATP concentrations.

**Data Analysis**

All data are reported as means ± S.E.M. In vivo blood pressure measurements, plasma histamine concentration, and left ventricular recovery of developed pressure were analyzed by two-way repeated measures analysis of variance (time and treatment) to determine whether there was a main effect of time, a main effect of treatment, or a time-treatment interaction. If global tests showed a main effect or interaction, post hoc analysis was performed using unpaired or paired analyses, as appropriate. Infarct size, risk region size, and left ventricular functional recoveries at 45 min of reperfusion in the isolated heart studies were compared using one-way analysis of variance followed by Student’s t test with the Bonferroni correction or an unpaired Student’s t test, as appropriate.

**Results**

**Radioligand Binding and cAMP Assays**

In vitro radioligand binding and cAMP assays using HEK 293 cell lines overexpressing mouse ARs demonstrated that CP-532,903 is a highly selective agonist of the mouse A3AR. In competition binding assays, CP-532,903 bound with ~100-fold higher affinity to the mouse A3AR compared to the mouse A1AR (Fig. 2). In HEK 293 cells overexpressing the mouse A1AR, CP-532,903 inhibited forskolin-stimulated cAMP production with greater than 200-fold higher potency compared with assays conducted with HEK 293 cells overexpressing the mouse A1AR (Fig. 3). CP-532,903 did not stimulate cAMP production in cells transfected with mouse A2A or A2BARs at concentrations as high as 10 μM, demonstrating that it has little agonist activity for mouse A2ARs (Fig. 3).

**Isolated Mouse Heart Studies**

**Hemodynamic Studies.** Preliminary studies were conducted with the Langendorff-perfused mouse heart model to examine the effect of CP-532,903 on baseline hemodynamic parameters. Mouse hearts (n = 4) were allowed to stabilize for 20 min (no pacing), and then increasing concentrations of CP-532,903 were administered into the perfusion buffer while heart rate, left ventricular pressure, and coronary flow were recorded for 5 min. The effect of the A1AR agonist 2-chloro-N6-cyclopentyladenosine (CCPA; 30 nM) was also tested. As shown in Fig. 4, CP-532,903 produced no changes in any of the hemodynamic parameters measured, whereas CCPA markedly decreased heart rate, +dP/dt, and −dP/dt. CCPA also tended to decrease coronary flow, but not significantly. These results demonstrate that administration of CP-532,903 at concentrations as high as 100 nM did not activate A1 or A2AARs in this model system.
Since it has been suggested that some A3AR agonists with limited selectivity (i.e., IB-MECA) may achieve cardioprotection by activating the A2AAR rather than the A3AR (Yang et al., 2003), in an additional series of experiments, we examined whether the A2AAR system is functional in A3KO mice by measuring coronary flow responses to the A2AAR agonist CGS 21680. As shown in Fig. 4, administration of CGS 21680 (100 nM) increased coronary flow in hearts from A3KO mice to a similar extent compared with wild-type hearts.

Ischemia/Reperfusion Studies. Administration of CP-532,903 concentration dependently improved functional recovery of hearts subjected to 20 min of global ischemia and 45 min of reperfusion (Fig. 5). At a concentration of 100 nM, developed pressure, $+dP/dt$, and $-dP/dt$ were improved from 47.5 ± 1.1 to 58.2 ± 1.2%, from 49.4 ± 1.3 to 61.3 ± 1.5%, and from 39.7 ± 1.3 to 50.5 ± 1.7% of baseline at 45 min of reperfusion, respectively. Treatment with 30 nM CP-532,903 provided no benefit in studies using hearts obtained from A3AR KO mice (Fig. 6). Noticeably, recovery of function during the first 10 min of reperfusion was impaired in A3KO compared with wild-type mice, suggesting that endogenous adenosine acting through native A3ARs may provide some degree of protection during ischemia/reperfusion injury.

We subsequently examined whether treatment with CP-532,903 provides protection against ischemia/reperfusion injury in hearts obtained from Kir6.2 KO mice. Kir6.2 KO mice lack functional $I_{KATP}$ in cardiac myocytes due to genetic disruption of $KCNJ11$ encoding the Kir6.2 pore-forming subunit of the sarcolemmal $K_{ATP}$ channel (Fig. 7) (Suzuki et al., 2002). For purposes of comparison, we also examined whether protection provided by IPC is functional in Kir6.2 KO mice.
KO hearts. As shown in Fig. 8, recovery of left ventricular developed pressure, \( dP/dt \), and \( dP/dt \) was not improved by treatment with 30 nM CP-532,903 in Kir6.2 KO hearts subjected to 20 min of global ischemia and 45 min of reperfusion. Postischemic functional recovery was also not improved by IPC induced by three 3-min occlusion/2-min reperfusion cycles, whereas the same IPC protocol improved all measures of contractile function of wild-type hearts from 45% to nearly 60% of preischemic levels (Fig. 9). Collectively, these results demonstrate that protection provided by activation of the A3AR as well as by IPC in isolated hearts is mediated by a mechanism involving the sarcolemmal KATP channel.

**In Vivo Infarction Studies**

**Ischemia/Reperfusion Studies.** In in vivo studies, infarct size was 59.2 ± 2.1% of the risk region in vehicle-treated mice subjected to 30 min of LAD occlusion and 24 h of reperfusion. Administration of 30 or 100 μg/kg CP-532,903 significantly reduced infarct size to 42.5 ± 2.3 and 39.0 ± 2.9%, respectively (Fig. 10). At a dose of 100 μg/kg, administration of CP-532,903 did not reduce infarct size in studies using A3AR KO mice (Fig. 10). The size of the risk region, which ranged from 37.4 ± 2.9 to 40.6 ± 2.4% of the left ventricle, was not significantly different among the five experimental groups.

**Hemodynamic and Plasma Histamine.** In parallel studies, we examined the effect of 100 μg/kg CP-532,903 on systemic hemodynamic parameters and plasma histamine levels. Although A3AR agonists do not alter hemodynamic variables in nonrodent species including humans, rabbits, and dogs (Auchampach et al., 1997b, 2003; van Troostenburg et al., 2004), activation of the A3AR evokes the release of mediators from mast cells in rodents, which indirectly causes hypotension (Hannon et al., 1995; Van Schaik et al., 1996; Ge et al., 2006). As shown in Fig. 10, administration of 100 μg/kg CP-532,903 produced a 30% decrease in mean arterial blood pressure that persisted for at least 45 min without changing heart rate (data not shown). Concomitantly, administration of CP-532,903 significantly increased plasma histamine levels, from 198 ± 22 nM at baseline to 3104 ± 559 nM 15 min after administration of the drug (Fig. 10). In A3KO mice, administration of CP-532,903 had no effect on blood pressure or plasma histamine levels.

**Electrophysiology Studies**

Our results obtained with Kir6.2 KO mouse suggest that the A3AR is functionally coupled to the sarcolemmal KATP channel. To test this hypothesis, whole-cell recordings were obtained from myocytes isolated from wild-type mice in the presence of CPX (500 nM) and ZM 241385 (500 nM), antagonists of mouse A1 and A2ARs, respectively (Kreckler et al., 2006). Basal whole-cell current was initially monitored for 20 min to allow for the diffusional exchange of ATP between the pipette solution and the intracellular milieu. Myocytes that exhibited spontaneous activation of outward current during this time period were discarded. Extracellular application of
CP-532,903 elicited an outward current that was blocked by glibenclamide (Fig. 11). This current was identified as IKATP. To confirm that the IKATP elicited by CP-532,903 was indeed due to activation of the A3AR, the experiments were repeated with myocytes isolated from A3KO mice. As shown in Fig. 11, the ability of CP-532,903 to elicit opening of the KATP channel was markedly attenuated. These results demonstrate a functional coupling between the A3AR and the KATP channel.

Mitochondrial Function Studies

Since it has been hypothesized that mitochondria may express a related isoform of the KATP channel that has yet to be molecularly identified (Hanley and Daut, 2005), we examined respiration and ATP synthesis capacity of mitochondria isolated from Kir6.2 KO mice to determine whether deletion of KCNJ11 affects normal function of cardiac mitochondria. Compared with wild-type mice, we observed no differences in state 2, 3, or 4 respiration, the respiratory control ratio, or ATP synthesis rates of cardiac mitochondria isolated from Kir6.2 KO mice (Fig. 12).

Discussion

We examined the cardioprotective effects of the A3AR agonist CP-532,903 in two mouse models of ischemia/reperfusion injury. We found that administration of CP-532,903 concentration-dependently improved functional recovery of isolated mouse hearts subjected to global ischemia and reperfusion and reduced the size of myocardial infarction in mice subjected to 30 min of coronary occlusion and 24 h of reperfusion. The protective effects of CP-532,903 were considerable, producing an ~40% reduction in infarct size and significant improvement in all parameters of posts ischemic functional recovery of isolated mouse hearts. In the isolated mouse heart studies, CP-532,903 was effective when used at low concentrations that did not influence A1AR-mediated bradycardia or A2A/A2BAR-mediated coronary dilation. The protection against injury provided by CP-532,903 was not apparent in isolated heart studies using hearts from A3KO mice or from Kir6.2 KO mice lacking expression of the pore-forming subunit of the sarcolemmal KATP channel. These observations demonstrate that CP-532,903 provides a direct cardioprotective effect in the ischemic myocardium by a mechanism involving activation of the A3AR and sarcolemmal KATP channels.

CP-532,903 is a member of a new series of N\textsuperscript{6}-benzylsubstituted adenosine 5'-N methylcarboxamide AR agonists developed by Hill and colleagues (DeNinno et al., 2003, 2006; Tracey et al., 2003) that resulted from a search for more selective human A3AR agonists. The unique structural feature of CP-532,903 as well as other members within this series is an amino substitution at the 3' position of the ribose ring rather than a hydroxyl group (Fig. 1). Unlike other members in this series, including CP-608,039, CP-532,903 has been reported to maintain relatively high A3AR selectivity in some nonhuman species including the rabbit (90-fold), leading to the selection of this compound as a potentially useful agent for study of the A3AR in preclinical animal models (DeNinno et al., 2003, 2006; Tracey et al., 2003). In the present investigation, we confirmed that CP-532,903 also displays high selectivity for the mouse A3AR. In radioligand binding assays, CP-532,903 bound with high affinity (K\textsubscript{i} = 9.6 nM) to the mouse A3AR and with 100-fold selectivity compared with its closest pharmacological relative the A1AR. The A3 versus A1AR selectivity of CP-532,903 in the mouse is comparable with that of the first generation A3AR agonists IB-MECA (68-fold) and CI-IB-MECA (210-fold; Ge et al., 2006). One important finding of our studies is that we observed that CP-532,903 exhibited very weak agonist activity for mouse A2A and A2BARs producing no stimulation up to a concentration of 10 \mu M (Fig. 3). This is a significant observation since it has been suggested that IB-MECA and CI-IB-MECA may stimulate A2A ARs with relatively high potency in some species (Murphree et al., 2002). Indeed, we have previously shown that IB-MECA stimulated cAMP production in HEK 293 cells transfected with the mouse A2A,AR with an EC\textsubscript{50} value of ~700 nM (Ge et al., 2006). Thus, CP-532,903 exhibits superior A3 versus A2A/A2BAR selectivity compared with currently available first generation A3AR agonists.

Tracey et al. (2003) have previously reported that CP-
532,903 effectively reduced infarct size in an isolated rabbit heart model of regional infarction. In these studies, pretreatment with CP-532,903 was shown to produce a maximal reduction in infarct size of 77% at a concentration of 150 nM and an EC50 value of −1 nM (Tracey et al., 2003). Pretreatment with CP-532,903 was also shown to reduce infarct size in an in vivo rabbit model of infarction at doses (0.25 and 1 mg/kg) that were devoid of hemodynamic effects (Tracey et al., 2003). Although CP-532,903 was shown to be an effective cardioprotective agent in this study, it remained uncertain whether it reduced ischemic injury via activation of the A3AR or via interaction with the other AR subtypes. This issue could not be addressed directly since selective A3AR agonists proven to be useful in the rabbit are not currently available. The results of the present investigation clearly demonstrate that the protection provided by CP-532,903 in both the isolated mouse heart model as well as the in vivo mouse model of infarction was completely lost in studies using A3AR KO mice, supporting the theory that CP-532,903 alleviates ischemic injury via the A3AR.

The inward rectifying K+ channel Kir6.2 is the pore-forming subunit of the KATP channel (Inagaki et al., 1995; Seino, 1999; Alekseev et al., 2005; Kane et al., 2005). In cardiac myocytes, Kir6.2 associates with the glibenclamide-sensitive sulfonylurea SUR2A subunit to form functional KATP channels in the sarcolemmal membrane (Inagaki et al., 1996; Alekseev et al., 2005; Kane et al., 2005). KATP channels, which are tightly coupled to the metabolic state of the cell, open in response to various stresses functionally shortening the duration of the action potential lessening time for calcium influx (Alekseev et al., 2005; Kane et al., 2005). Thus, the KATP channel serves to adjust cellular excitability to match metabolic demand. With the aid of Kir6.2 KO mice, the sarcolemmal KATP channel has been shown to importantly participate in normal stress responses, including IPC (Suzuki et al., 2002; Hanley and Daut, 2005; Kane et al., 2005). Although it remains controversial, a related isoform of the KATP channel has also been proposed to be expressed in mitochondria (Hanley and Daut, 2005). Similar to the sarcolemmal KATP channel, the mitochondrial KATP channel is thought to open in response to metabolic stress, thereby preserving mitochondrial integrity and reducing apoptosis secondary to changes caused by increased potassium influx. We and others (Tracey et al., 1998; Thourani et al., 1999; Auchampach et al., 2003) have previously observed that protection provided by A3AR agonists is blocked by coadministration of glibenclamide, which equally inhibits both isoforms of the KATP channel. However, the relative importance of the two KATP channel isoforms in A3AR-mediated cardioprotection remained unknown. In the present investigation, we have shown that, like IPC, administration of CP-532,903 does not provide cardioprotection in the isolated heart model using Kir6.2 KO mice confirmed to lack functional sarcolemmal KATP. We have also provided evidence suggesting that the A3AR is expressed in cardiomyocytes and that it couples to opening of the sarcolemmal KATP channel using electrophysiological techniques. Suzuki et al. (2002) have previously shown that the mitochondrial KATP channel is functional in Kir6.2 KO mice, based on diazoxide-induced changes in flavoprotein oxidation. We have also shown in the present investigation that mitochondrial respiration and ATP-synthesizing capacity of mitochondria from Kir6.2 KO mice are...
Thus, our data suggest that CP-532,903 provided a direct cardioprotective effect in the isolated heart model via activation of the sarcolemmal K<sub>ATP</sub> channel, rather than via the putative mitochondrial K<sub>ATP</sub> channel. The data from the isolated heart experiments indicate that CP-532,903 produced a direct cardioprotective effect involving the sarcolemmal K<sub>ATP</sub> channel. However, it is possible that CP-532,903 reduced infarct size in vivo by additional or alternative mechanisms. Administration of CP-532,903 increased plasma histamine levels and produced a 30% decrease in mean arterial blood pressure, responses that were not observed in A<sub>3</sub>AR KO mice. Thus, improvement in the oxygen supply-demand balance or depletion of mast cell contents may have contributed to the reduction in infarct size in the in vivo model, although we think these potential mechanisms are unlikely based on previous work performed by our laboratory (Ge et al., 2006). Since it has been shown that A<sub>3</sub>AR agonists including CP-532,903 reduce infarct size when administered at the time of reperfusion (Auchampach et al., 2003; Tracey et al., 2003), it is also possible that CP-532,903 protected against infarction in the present investigation by reducing reperfusion-mediated injury. Even though CP-532,903 was administered 10 min before the ischemic period in our studies, it was likely present during the initial phase of reperfusion since hemodynamic responses to CP-532,903 persisted for over 45 min. Numerous studies in various models of inflammation have suggested that A<sub>3</sub>AR activation suppresses inflammatory responses and may regulate neutrophil-mediated injury (Hasko and Cronstein, 2004). Thus, CP-532,903 may have acted, in part, by suppressing injury caused by reperfusion-induced inflammation. In support of this theory, we have shown in preliminary experiments that CI-IB-MECA does not reduce infarct size.
when administered at the time of reperfusion in bone marrow chimeric mice lacking the expression of A2ARs in bone marrow-derived cells (Ge et al., 2004). Although the results of the present investigation clearly implicate the involvement of the A2AR, additional studies are required to investigate the multiple different mechanisms by which CP-532,903 effectively limits infarct size under in vivo conditions and to determine the relative contribution of the sarcomelmal K\textsubscript{ATP} channel.

In summary, CP-532,903 has been shown to be a highly selective agonist for the mouse A3AR with improved selectivity versus the A3AR subtypes. Thus, CP-532,903 should be useful for discerning the biological role of the A3AR in mice. The results further confirm that CP-532,903 exerts a direct protective effect on the ischemic myocardium by activation of the A3AR by a mechanism that involves opening the sarcomelial K\textsubscript{ATP} channel. The importance of this direct cardioprotective action under in vivo circumstances awaits further investigation. Finally, the results of the present investigation support the contention that A3AR agonists may be useful agents for treating acute ischemia/reperfusion injury.

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References


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