Cardiac and vascular K_{ATP} channels in rats are activated by endogenous epoxyeicosatrienoic acids through different mechanisms

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We have reported that epoxyeicosatrienoic acids (EETs), the cytochrome P450 (CYP) epoxygenase metabolites of arachidonic acid (AA), are potent sarcolemmal ATP-sensitive K^+ (K_{ATP}) channel activators. However, activation of cardiac and vascular K_{ATP} channels by endogenously produced EETs under physiological intracellular conditions has not been demonstrated and direct comparison of the mechanisms whereby EETs activate the K_{ATP} channels in cardiac myocytes *versus* vascular smooth muscle cells has not been made. In this study, we examined the effects of AA on K_{ATP} channels in freshly isolated cardiac myocytes from rats, wild-type (WT) and transgenic mice overexpressing CYP2J2 cDNA, and mesenteric arterial smooth muscle cells from rats. We also compared the activation of cardiac and vascular K_{ATP} channels by extracellularly and intracellularly applied 11,12-EET. We found that 1 μM AA enhanced K_{ATP} channel activities in both cardiac and vascular smooth muscle cells, and the AA effects were inhibited by preincubation with CYP epoxygenase inhibitors. Baseline cardiac K_{ATP} current densities in CYP2J2 transgenic mice were 190% higher than those of WT mice, and both were reduced to similar levels by CYP epoxygenase inhibition. Western blot analysis showed that expression of Kir6.2 and SUR2A was similar between WT and CYP2J2 transgenic hearts. 11,12-EET (5 μM) applied intracellularly enhanced the K_{ATP} currents by 850% in cardiac myocytes, but had no effect in vascular smooth muscle cells. In contrast, 11,12-EET (5 μM) applied extracellularly increased K_{ATP} currents by 520% in mesenteric arterial smooth muscle cells, but by only 209% in cardiac myocytes. Preincubation with 100 μM *m*-iodobenzylguanidine or 5 μM myristoylated PKI amide did not alter the activation of cardiac K_{ATP} channels by 5 μM 11,12-EET, but significantly inhibited activation of vascular K_{ATP} channels. Moreover, EET only enhanced the inward component of cardiac K_{ATP} currents, but activated both the inward and outward components of vascular K_{ATP} currents. Our results indicate that endogenously derived CYP metabolites of AA potently activate cardiac and vascular K_{ATP} channels. EETs regulate cardiac electrophysiology and vascular tone by K_{ATP} channel activation, albeit through different mechanisms: the cardiac K_{ATP} channels are directly activated by EETs, whereas activation of the vascular K_{ATP} channels by EETs is protein kinase A dependent.

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Sarcolemmal ATP-sensitive K^+ (K_{ATP}) channels are hetero-octameric complexes, composed of four pore-forming subunits (Kir6.x) and four sulphonylurea receptor (SUR) subunits (Aguilar-Bryan *et al.* 1998). K_{ATP} channels are robustly expressed in cardiac myocytes at 1–10 channels per square micrometre (Yokoshiki *et al.* 1998), but are present at lower density in vascular smooth muscle cells (0.1–0.2 channels per square micometre), populating both conduit and resistance vessels (Quayle *et al.* 1997). K_{ATP} channels are inhibited by intracellular ATP, but can be activated during ischaemia and hypoxia in response to changes of the intracellular metabolic state. Cardiac K_{ATP} channels are activated by intracellular nucleotide diphosphates (Tung & Kurachi, 1991), by

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protons (Koyano *et al.* 1993), and by G protein-mediated signalling pathways, including protein kinase A (PKA) (Lin *et al.* 2000), protein kinase C (Ito *et al.* 2001) and G-protein subunits (Terzic *et al.* 1994). Opening of the cardiac K_{ATP} channel leads to shortening of the cardiac action potential, reducing intracellular Ca^{2+} overload, and is thought to be a crucial mediator of cardiac preconditioning and postischaemic cardiac protection (Seino & Miki, 2003). Activation of the vascular K_{ATP} channel leads to membrane hyperpolarization, reduces Ca^{2+} influx, and is a key determinant of vascular tone (Yokoshiki *et al.* 1998). Studies with Kir6.2 knock-out and Kir6.2 overexpressor mice have demonstrated that cardiac K_{ATP} channels are important regulators of cardiac electrophysiology and contractility (Suzuki *et al.* 2001; Rajashree *et al.* 2002; Saito *et al.* 2005), and are critical elements for cardiac adaptation to stress (Zingman *et al.* 2002; Hodgson *et al.* 2003). Mice with ablation of the Kir6.1 gene, which encodes vascular K_{ATP} channels, developed spontaneous coronary spasm that leads to lethal atrioventricular block and sudden death (Miki *et al.* 2002). In addition, K_{ATP} channels are implicated in cardiac arrhythmogenesis (Liu *et al.* 2004) and in the development of dilated cardiomyopathy (Bienengraeber *et al.* 2004).

Cytochrome P450 (CYP) enzymes are widely distributed in mammalian tissues and play crucial roles in the metabolism of endogenous substrates and exogenous chemicals. More than 500 CYP genes and 78 CYP families have been identified (Roman, 2002). The CYP2Js are the primary CYP epoxygenase isoforms in cardiac myocytes, whereas the CYP2Cs are dominant in vascular beds. These enzymes convert arachidonic acid (AA) into four epoxyeicosatrienoic acid (EET) regioisomers: 5,6-, 8,9-, 11,12- and 14,15-EET (Wu *et al.* 1996; Zeldin, 2001; Roman, 2002). EETs are abundant in the rat heart, with 70 ng of total EETs per gram of heart (Wu *et al.* 1997). Most of AA is stored in membrane phospholipids, and upon phospholipase A2 activation can be released and converted into EETs (Wu *et al.* 1997; Zeldin, 2001). Moreover, EETs are rapidly taken up by cardiac myocytes and incorporated into membrane phospholipids, which serve as an important EET source (Lee *et al.* 1999). These mechanisms contribute to the significant increase in cardiac EET concentrations during ischaemia and reperfusion (Nithipatikom *et al.* 2001).

EETs are known to regulate multiple cellular functions including inflammation (Node *et al.* 1999), cell proliferation (Potente *et al.* 2002), vasoreactivity (Zhang *et al.* 2001), and cardiac protection from ischaemia/reperfusion injury (Wu *et al.* 1997; Seubert *et al.* 2004; Gross *et al.* 2005; Nithipatikom *et al.* 2006). EETs are ion channel regulators, modulating the activities of vascular smooth muscle Ca^{2+}-activated K^+ channels (Campbell *et al.* 1996; Lu *et al.* 2001*b*; Weston *et al.* 2005), cardiac Na^+ channels (Lee *et al.* 1999), cardiac L-type Ca^{2+} channels (Xiao *et al.* 1998, 2004; Chen *et al.* 1999), epithelial Na^+ channels (Wei *et al.* 2004), transient receptor potential (TRP) channels (Watanabe *et al.* 2003) and K_{ATP} channels including those in blood vessels (Ye *et al.* 2005), in sarcolemma (Lu *et al.* 2001*a*) and in cardiac mitochondria (Seubert *et al.* 2004). Most of these studies used exogenously applied EETs. Whether endogenously produced EETs would activate K_{ATP} channels in cardiovascular cells under physiological conditions is unknown.

The four EET regioisomers are equipotent activators of cardiac and vascular K_{ATP} channels (Lu *et al.* 2002; Ye *et al.* 2005). Activation of K_{ATP} channels by EETs may be mediated by different mechanisms, which may depend on the specific cellular organelle and cell type. In rat mesenteric arterial smooth muscle cells, activation of K_{ATP} channels by EETs was dependent on PKA activity (Ye *et al.* 2005, 2006). In cardiac mitochondria, activation of K_{ATP} channels by EETs is mediated through the p42/p44 mitogen activated protein (MAP) kinase signalling pathway (Seubert *et al.* 2004). In rat cardiac ventricular myocytes, activation of K_{ATP} channels by EETs involves direct interactions with specific positively charged residues, R192/R195, on the C-terminus of the Kir6.2 subunits, resulting in reduced channel sensitivity to ATP (Lu *et al.* 2005). However, it is unknown whether the PKA signalling pathway is also involved in the activation of cardiac K_{ATP} channels by EETs. In the present study, we tested the hypothesis that CYP epoxygenase activity is important for the maintenance of K_{ATP} channel function under physiological conditions and that endogenously derived EETs from AA metabolism could activate K_{ATP} channels in cardiac myocytes and in vascular smooth muscle cells. We further hypothesized that activation of the cardiac and vascular K_{ATP} channels by EETs is mediated through different signalling mechanisms. We compared the effects of 11,12-EET with another K^+ channel opener (pinacidil) on cardiac K_{ATP} channel activation, and also the difference between cardiac and vascular K_{ATP} channel activities in response to 11,12-EET applied intracellularly and extracellularly. We found that AA activated K_{ATP} channels through CYP epoxygenase conversion to EETs in cardiac myocytes from normal rats, wild-type (WT) mice, transgenic mice overexpressing CYP2J2 cDNA, and rat mesenteric arterial smooth muscle cells. Cardiac K_{ATP} channels were preferentially activated by intracellularly applied EETs, whereas vascular K_{ATP} channels were only activated when EETs were applied extracellularly. In addition, PKA activity was crucial for EET-mediated K_{ATP} channel activation in vascular smooth muscle cells, but not in cardiac ventricular myocytes.

Methods

Animals

The use of animals and the procedures were approved by the Institutional Animal Care and Use Committee at Mayo Clinic. Male Sprague-Dawley rats (200–250 g) were used for isolation of cardiac myocytes and mesenteric arterial smooth muscle cells. Transgenic mice with cardiac myocyte-specific overexpression of CYP2J2 and age/sex-matched WT controls were bred and genotyped as previously described (Seubert *et al.* 2004; Xiao *et al.* 2004).

Isolation of cardiac ventricular myocytes

Single ventricular myocytes were isolated enzymatically as previously described (Lu *et al.* 1999). Briefly, male Sprague-Dawley rats (200–250 g body weight) were anaesthetized with pentobarbital (100 mg kg^{-1} I.P.). The rat hearts were rapidly excised and perfused with nominally Ca^{2+}-free Tyrode solution using a modified Langendorff apparatus at 8 ml min^{-1} for 5 min. The nominally Ca^{2+}-free Tyrode solution contained (mM): NaCl 138, KCl 4.5, $MgCl_2$ 0.5, Na_2HPO_4 0.33, glucose 5.5, Hepes 10, and bovine serum albumin 0.1% (w/v), adjusted to pH 7.4 with NaOH. The perfusate was switched to a nominally Ca^{2+}-free Tyrode solution containing 0.6 mg ml^{-1} collagenase (Worthington, CLS-2, 347 units mg^{-1}) for 10 min. The ventricle was transferred into fresh 0.6 mg ml^{-1} collagenase solution, cut into small pieces ($\sim$2 mm^3) and incubated at room temperature (22°C) for 5 min. Dissociated cells were filtered through a medium mesh, centrifuged and stored in KB solution which contained (mM): KOH 70, KCl 40, L-glutamic acid 50, taurine 20, $MgCl_2$ 0.5, K_2HPO_4 1, EGTA 0.5, Hepes 10, creatine 5, pyruvic acid 5, and Na_2ATP 5, adjusted to pH 7.38 with KOH. Mice were anaesthetized by isofluorane inhalation, and isolated hearts were retrogradely perfused on the Langendorff apparatus (1 ml min^{-1}) for 5 min with Krebs-Ringer solution containing (mM): NaCl 35, KCl 4.75, KH_2PO_4 1.2, Na_2HPO_4 16, sucrose 134, Hepes 10, glucose 10, $NaHCO_3$ 25, pH 7.4 with NaOH and equilibrated with 5% CO_2. Then, the mouse hearts were perfused with Krebs-Ringer solution containing 1 mg ml^{-1} collagenase for another 8 min. Dissociated cells were stored in KB solution and used within 24 h.

Isolation of mesenteric arterial smooth muscle cells

Mesenteric smooth muscle cells were dissociated enzymatically as previously reported (Ye *et al.* 2005). Third- and fourth-ordered branches of rat mesenteric arteries were carefully dissected. The small arteries were then placed in 1 ml of physiological saline solution which contained (mM): NaCl 145, KCl 4, $CaCl_2$ 0.05, $MgCl_2$ 1, Hepes 10, glucose 10, pH 7.2 with bovine serum albumin 0.1% (w/v). After 10 min incubation at 37°C in a shaking water bath, the vessels were treated with 1.75 mg papain and 1.25 mg dithiothreitol in 1 ml saline solution for 10 min. The vessels were further digested with 1.25 mg collagenase and 1.25 mg of trypsin inhibitor in 1 ml saline solution at 37°C for 10 min, then washed three times with 1 ml aliquots of saline solutions, and gently triturated with a fire-polished glass pipette until the cells were completely dissociated.

Whole-cell K_{ATP} current recordings

Whole-cell K^+ currents in freshly isolated cardiac ventricular myocytes and mesenteric arterial smooth muscle cells were recorded using an Axopatch 200B integrating amplifier (Axon Instruments, Union City, CA, USA), filtered at 2 kHz and digitized at 50 kHz. In cardiac myocytes, K^+ currents were elicited at test potentials (TP) from −120 mV to +40 mV at 10 mV increments from a holding potential (HP) of 0 mV to inactivate Na^+ and Ca^{2+} channels. To further eliminate Na^+ and Ca^{2+} current contaminations, the following bath solution was used (mM): NaCl 20, KCl 4.5, choline chloride 130, $CaCl_2$ 1, $CoCl_2$ 2, $MgCl_2$ 2, Hepes 10, and glucose 5.5, pH 7.4, as previously reported (Lu *et al.* 1999). Pipette resistance was 0.5–0.8 MΩ when filled with the pipette solution which contained (mM): KCl 140, $CaCl_2$ 0.465 (200 nM free Ca^{2+}), $MgCl_2$ 0.5, Na_2ATP 5, Na_2GTP 0.5, Hepes 1, EGTA 1, pH 7.3. Cardiac K_{ATP} currents were obtained as the HMR-1098-sensitive (3 μM), or the glyburide-sensitive (5 μM) K^+ currents by digital subtraction. In vascular smooth muscle cells, K^+ currents were recorded (TP from −100 mV to 40 mV, HP = 0 mV) in symmetrical K^+ with the bath solution containing (mM) KCl 140, $MgCl_2$ 1, $CaCl_2$ 0.1, Hepes 10, glucose 10.0, IBTX 0.1, pH 7.4, and the pipette solution containing (mM) KCl 110, KOH 30, $MgCl_2$ 1, $CaCl_2$ 1 (20 nM free Ca^{2+}), Na_2ATP 0.1, Na_2ADP 0.1 and Na_2GTP 0.5, Hepes 10, EGTA 10, pH 7.35. Vascular K_{ATP} currents were obtained as the glyburide-sensitive (10 μM) K^+ currents. Current–voltage (*I–V*) relationships were fitted using equations previously described (Lu *et al.* 2001*a*).

Single channel recordings

Single channel K_{ATP} and the strong inwardly rectifying K^+ (I_{K1}) currents in inside-out membrane patches of cardiac myocytes (TP of −60 and +60 mV) were recorded in symmetrical K^+ (140 mM) as previously described (Lu *et al.* 2001*a*). The output signals were filtered with an eight-pole Bessel filter (902 LPF, Frequency Devices Inc., Haverhill, MA, USA) at 1 kHz and digitized at 20 kHz.

Patch pipettes were coated with Sylgard 184 (Dow Corning, Midland, MI, USA) and had a typical tip resistance of 10 MΩ when filled with the pipette solution that contained (mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 0.1, Hepes 10. pH 7.4. The bath solution contained (mM): KCl 70, L-aspartic acid monopotassium salt 70, EGTA 1, Hepes 10, $MgCl_2$ 1, pH to 7.35. Data were acquired and further analysed using pCLAMP 8 software (Axon Instruments). All experiments were conducted at room temperature (22°C).

Western blot analysis

Polyclonal rabbit anti-Kir6.2 and anti-SUR2A antibodies were custom prepared against synthetic peptides corresponding to antigenic sites in rat Kir6.2 (16-PLRKRSVAVAKAKPKFSISPDSLS-39) and SUR2A (263-LKEAYEEQKKKAADHPNRTPS-284). Kir6.2 and SUR2A antibodies bind to cardiac proteins of 40 kDa and 140 kDa, respectively, consistent with previous results (Crawford *et al.* 2002; Morrissey *et al.* 2005). Kir6.2 and SUR2A protein expression in hearts were determined using Western blots. Left ventricles from three pairs of WT and CYP2J2 transgenic mice were homogenized in 300 μl of T-PER tissue protein-extraction reagent containing protease inhibitors (Pierce, Biotechnology Inc.), resolved on a 4–15% Tris-HCl polyacrylamide gradient gel, and transferred electrophoretically onto polyvinylidene difluoride membranes (Wang *et al.* 2005). The membranes were blocked for 2 h with 10% milk in phosphate-buffered saline (PBS), followed by incubation with rabbit anti-Kir6.2 or anti-SUR antibodies (1 : 300 dilution). After extensive washes with 0.1% PBS–Tween, the primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies, and the signals were developed by an Immune-Star HRP Chemiluminescence Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Optical densities of the bands were analysed using Scion Image software (Scion, Frederick, MD, USA).

Immunoblotting of heart microsomal fractions to confirm the presence of the CYP2J2 transgene was performed using polyclonal antibodies against the recombinant human CYP2J2 protein (anti-CYP2J2rec) and against the CYP2J2-specific peptide HMDQNF-GNRPVTPMR (anti-CYP2J2pep1) as previously described (Wu *et al.* 1996; King *et al.* 2002).

Chemicals

11,12-EET was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA), prepared in 100% ethanol (5 mM) and stored at −80°C. Myristoylated PKI amide and β-diethyl-aminoethyldiphenylpropylacetate (SKF525A) were purchased from BIOMOL (Plymouth Meeting, PA, USA). HMR-1098 was a gift from Aventis Pharmaceuticals Co. (Frankfurt, Germany). Unless otherwise mentioned, all other chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Statistical analysis

Data were presented as the mean ± S.E.M. Student's *t* test was used to compare data between two groups. One-way ANOVA followed by Tukey's test was used to compare data from multiple groups using SigmaStat software (Systat Software Inc., Point Richmond, CA, USA).

Results

Effects of AA on K_{ATP} channels in rat cardiac ventricular myocytes

In freshly isolated rat ventricular myocytes, whole-cell K^+ currents (HP = 0 mV, TP from −120 mV to +20 mV) were activated on exposure to 1 μM AA, reaching a maximal effect in 10–15 min without altering the current reversal potential (E_K). The effects of AA were blocked by 3 μM HMR-1098, a specific sarcolemmal K_{ATP} channel inhibitor that was added after the AA effects have reached steady state. Tracings of a typical recording are shown in Fig. 1*A*, and the K^+ current *I–V* relationships before and after application of AA and HMR-1098 are plotted in Fig. 1*B*. The HMR-sensitive K^+ currents, obtained by digital subtraction of the residual currents after exposure to HMR-1098 (3 μM) from the total K^+ currents, are defined as the cardiac K_{ATP} currents and shown in Fig. 1*C*. Group data summarizing the effects of AA on the *I–V* relationships of cardiac K_{ATP} channels are shown in Fig. 1*D*. Interestingly, AA significantly increased cardiac K_{ATP} currents at all voltages negative to E_K; for example, at a TP of −100 mV, AA increased the K_{ATP} currents from a baseline of -4.50 ± 0.66 pA pF^{-1} to -14.37 ± 6.18 pA pF^{-1} ($n = 5$, $P < 0.05$). In contrast, AA had no effects on K_{ATP} currents at voltages positive to E_K; for example, at a TP of −30 mV, baseline K_{ATP} current density was 0.38 ± 0.02 pA pF^{-1} and was 0.66 ± 0.76 pA pF^{-1} after exposure to AA ($n = 5$, N.S.). Hence, the effects of AA on cardiac K_{ATP} channels were voltage dependent, and AA activated only the inward but not the outward currents. These findings confirmed our previous results on single K_{ATP} channel recordings (Lu *et al.* 2001*a*). However, after incubation with 10 μM miconazole or 5 μM SKF525A (60 min), which are CYP epoxygenase inhibitors that block the conversion of AA to EETs, the baseline cardiac K_{ATP} currents were significantly reduced to -2.65 ± 0.11 pA pF^{-1} (TP = −100 mV, $n = 6$, $P < 0.05$ *versus* control), and AA failed to activate K_{ATP} currents (-2.10 ± 0.91 pA pF^{-1}, $n = 5$, N.S. *versus*

baseline) (Fig. 1*D*). These results indicate that the effects of AA on the cardiac K_{ATP} currents are mainly mediated through metabolites of CYP epoxygenase.

We compared the effects of AA with pinacidil, a K^+ channel opener, on cardiac K_{ATP} channel activation. Raw tracings of total K^+ currents at baseline, with pinacidil (100 μM), and then with HMR-1098 (3 μM) are shown in Fig. 2*A*, while the corresponding *I–V* relationships are plotted in Fig. 2*B*. Figure 2*C* shows the HMR-1098-sensitive K_{ATP} currents from the same recordings of Fig. 2*A* after digital subtraction. Pinacidil increased both inward (at TP of −100 mV, from -6.7 ± 2.3 pA pF^{-1} at baseline to -32.4 ± 12.0 pA pF^{-1}, $n = 3$, $P < 0.05$) and outward K_{ATP} currents (at TP of −30 mV, from -1.4 ± 0.6 pA pF^{-1} at baseline to 11.5 ± 5.3 pA pF^{-1}, $n = 3$, $P < 0.05$). These results are similar to previous reports that pinacidil activates both the inward and outward components of cardiac K_{ATP} currents (Terzic *et al.* 1995; Lawson, 2000); hence, the CYP epoxygenase metabolites of AA are unique K_{ATP} channel activators.

To further confirm the unique voltage dependence of K_{ATP} channel activation by the CYP epoxygenase metabolites of AA in cardiac myocytes, we examined the effects of 11,12-EET (5 μM) on inside-out single K_{ATP} and I_{K1} channel activities. In the presence of symmetrical K^+ (140 mM), the single K_{ATP} current amplitude was −5 pA at −60 mV and +2.5 pA at +60 mV, similar to what we have previously described (Lu *et al.* 2001*a*) (Fig. 3*A*). Opening probability (P_o) of K_{ATP} channels at baseline (0 mM ATP)

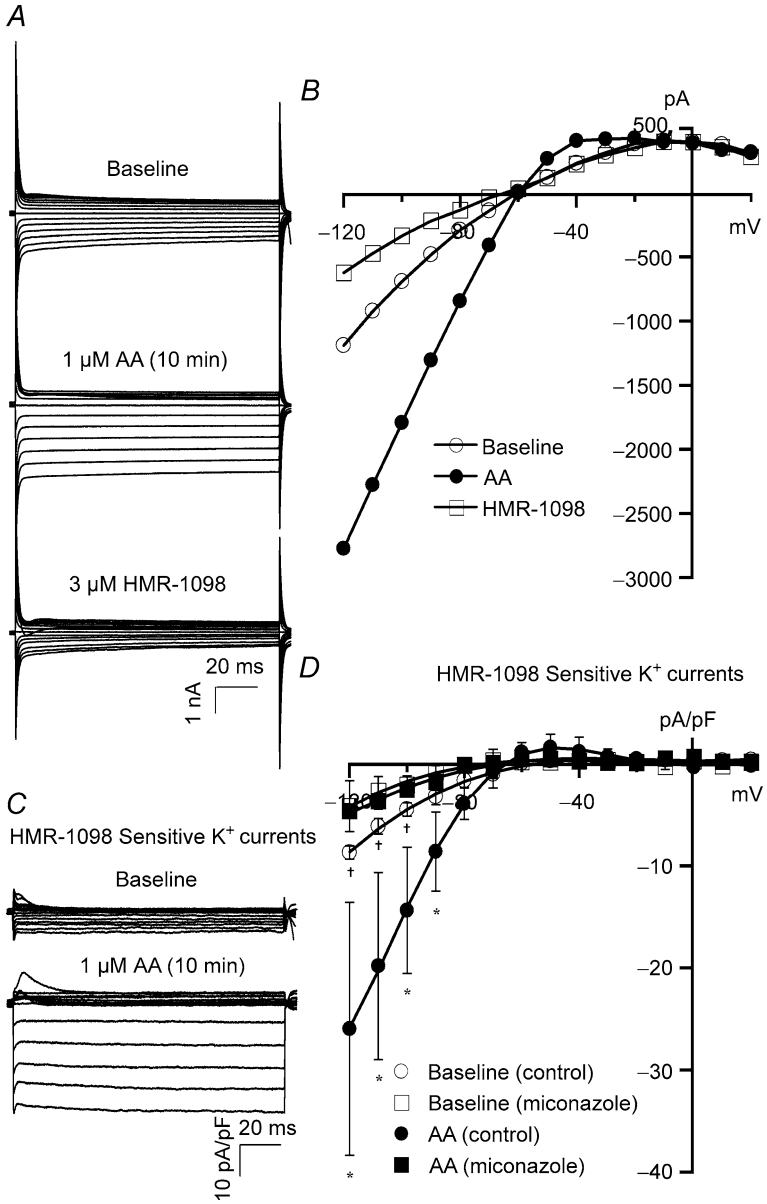

Figure 1. Effects of AA metabolites on cardiac K_{ATP} channel activities in rat ventricular myocytes
A, whole-cell total K^+ currents were elicited from −120 mV to +20 mV at a holding potential of 0 mV at increments of 10 mV in control rat ventricular myocytes. K^+ currents were activated by 1 μM AA, reaching steady state in 10 min, and then exposed to 3 μM HMR-1098. *B*, *I–V* relationships of total K^+ currents at baseline, in response to 1 μM AA, and 3 μM HMR-1098. *C*, HMR-1098-sensitive K^+ currents (cardiac K_{ATP} currents) were obtained from the current tracings in *A* by digital subtraction of the residual currents after exposure to HMR-1098 (3 μM) from the total K^+ currents. K_{ATP} currents were activated by AA. *D*, the K_{ATP} channel *I–V* relationships show inward rectification with a reversal potential of −70 mV. AA (1 μM) enhanced K_{ATP} currents at all voltages negative to reversal potential (inward currents). Treatment with 10 μM miconazole reduced K_{ATP} current densities at baseline and abolished the response to AA. Each point represents the mean ± S.E.M. $^*P < 0.05$ for AA *versus* baseline ($n = 6$). †$P < 0.05$ for baseline currents with *versus* without miconazole treatment ($n = 5$).

was 0.76 ± 0.12 at -60 mV and 0.23 ± 0.14 at $+60$ mV ($n = 3$), and on exposure to 1 mM ATP was reduced to 0.08 ± 0.05 at -60 mV ($n = 3$, $P < 0.05$ *versus* baseline) and to 0.05 ± 0.03 at $+60$ mV ($n = 3$, $P < 0.05$ *versus* baseline). However, in the presence of 1 mM ATP, application of 5 μM 11,12-EET significantly activated the K_{ATP} channels at $-$ 60 mV with a robust P_o of 0.42 ± 0.06 ($n = 3$, $P < 0.05$ *versus* baseline and 1 mM ATP alone). In contrast, there was no change in P_o at $+60$ mV by 11,12-EET (Fig. 3*A*). Hence, 11,12-EET activated specifically the inward component of K_{ATP} currents, converting the channel rectification properties from weakly inward to strongly inward. In the presence of symmetrical K^+ (140 mM), the single cardiac I_{K1} current amplitudes were -1.0 pA at -60 mV and $+0.2$ pA at $+60$ mV, and exposure to 5 μM 11,12-EET had no effect on channel activity, which was completely blocked by 1 mM $BaCl_2$ (Sakmann & Trube, 1984; Masuda & Sperelakis, 1993; Nakamura *et al.* 1998) (Fig. 3*B*). Baseline P_o of I_{K1} was 0.35 ± 0.14 at -60 mV and 0.03 ± 0.02 at $+60$ mV ($n = 2$). There was no change in P_o by 5 μM 11,12-EET ($n = 2$, 0.3 ± 0.1 at -60 mV and 0.003 ± 0.002 at + 60 mV, N.S. *versus* baseline). Group data on the effects of 11,12-EET on K_{ATP} and I_{K1} channel P_o are summarized in bar graphs (Fig. 3, lower panels). These results indicate that enhancement of inward rectifying K^+ currents by the AA metabolites of CYP epoxygenase pathway is mediated through the activation of K_{ATP} and not I_{K1} channels in rat ventricular myocytes.

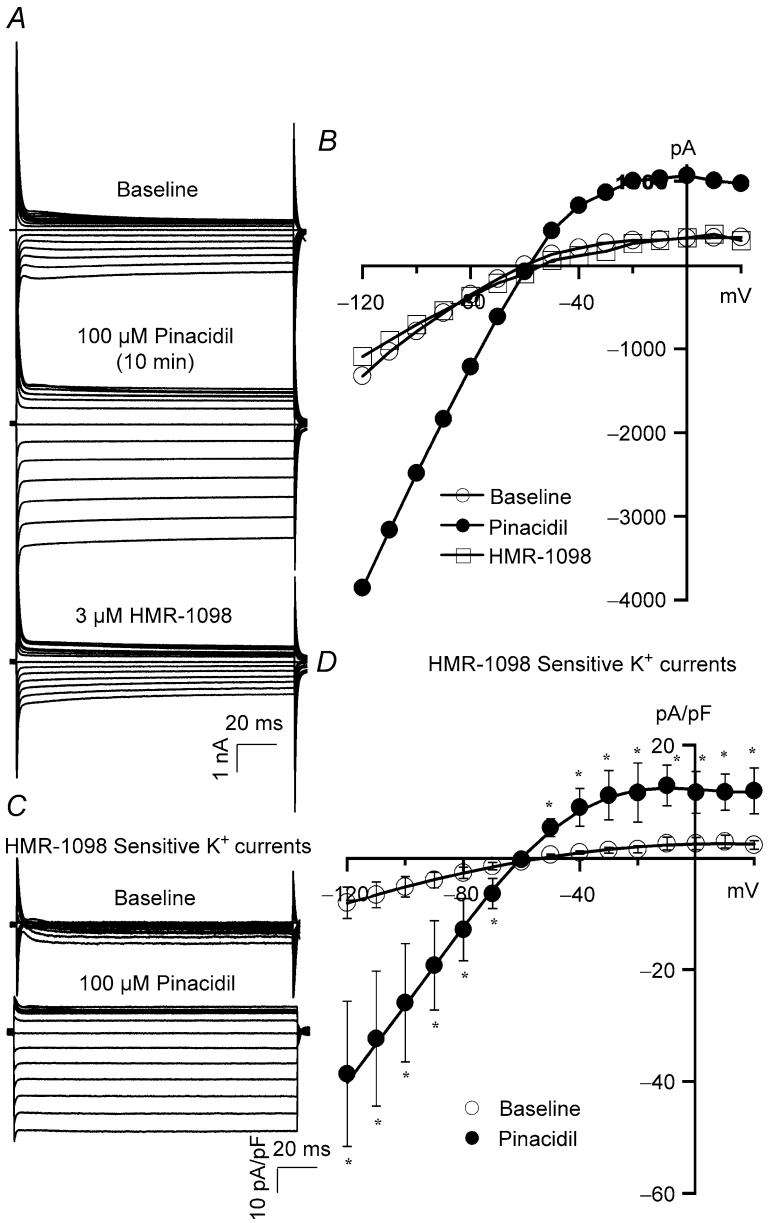

Figure 2. Effects of pinacidil on cardiac K_{ATP} channel activities
Whole-cell total K^+ currents (*A*) and the *I–V* relationships (*B*), in a rat ventricular myocyte, before and after exposure to 100 μM pinacidil, and then 3 μM HMR-1098, using the same protocol as described in Fig. 1. K_{ATP} currents (*C*) and *I–V* curves (*D*) were obtained from the current tracings of *A* by subtraction of the HMR-1098-insensitive K^+ currents. Unlike the effect of AA, 100 μM pinacidil enhanced both the inward and outward K_{ATP} currents. $^*P < 0.05$ *versus* baseline ($n = 3$).

Effects of AA on rat vascular K_{ATP} channels in mesenteric arterial smooth muscle cells

Figure 4*A* (left column) shows recordings of whole-cell total K^+ currents (TP = −100 mV to +40 mV, HP = 0 mV), from rat mesenteric arterial smooth muscle cells at baseline, with 1 μM AA, and then 10 μM glyburide. Vascular K_{ATP} currents were obtained by digital subtraction of the glyburide-insensitive K^+ currents (Fig. 4*A*, right column). Vascular K_{ATP} current densities were enhanced from -1.09 ± 0.35 pA pF^{-1} to -3.54 ± 0.78 pA pF^{-1} by 1 μM AA (TP = −100 mV, $n = 6$, $P < 0.05$ *versus* baseline). After incubation with 5 μM SKF525A (60 min), AA failed to activate vascular K_{ATP} channels (Fig. 4*B*). Figure 4*C* shows the vascular K_{ATP} channel *I–V* relationships in response to 1 μM AA, with and without pretreatment with SKF525A. AA (1 μM) only had small but significant effects on vascular K_{ATP} channels at negative voltages, and the effects were completely abolished by SKF525A. These results indicate that AA could activate vascular K_{ATP} channels through CYP metabolites, but the response is modest compared to that in cardiac ventricular myocytes.

Effects of AA on cardiac K_{ATP} channels in transgenic mice overexpressing CYP2J2

To further determine the role of CYP epoxygenase on cardiac K_{ATP} channel activation, we examined the effects of AA on cardiac myocytes from transgenic mice overexpressing the CYP2J2 gene. Figure 5*A* and *B* shows the HMR-1098-sensitive K_{ATP} channels from WT and CYP2J2 transgenic mice. The baseline cardiac

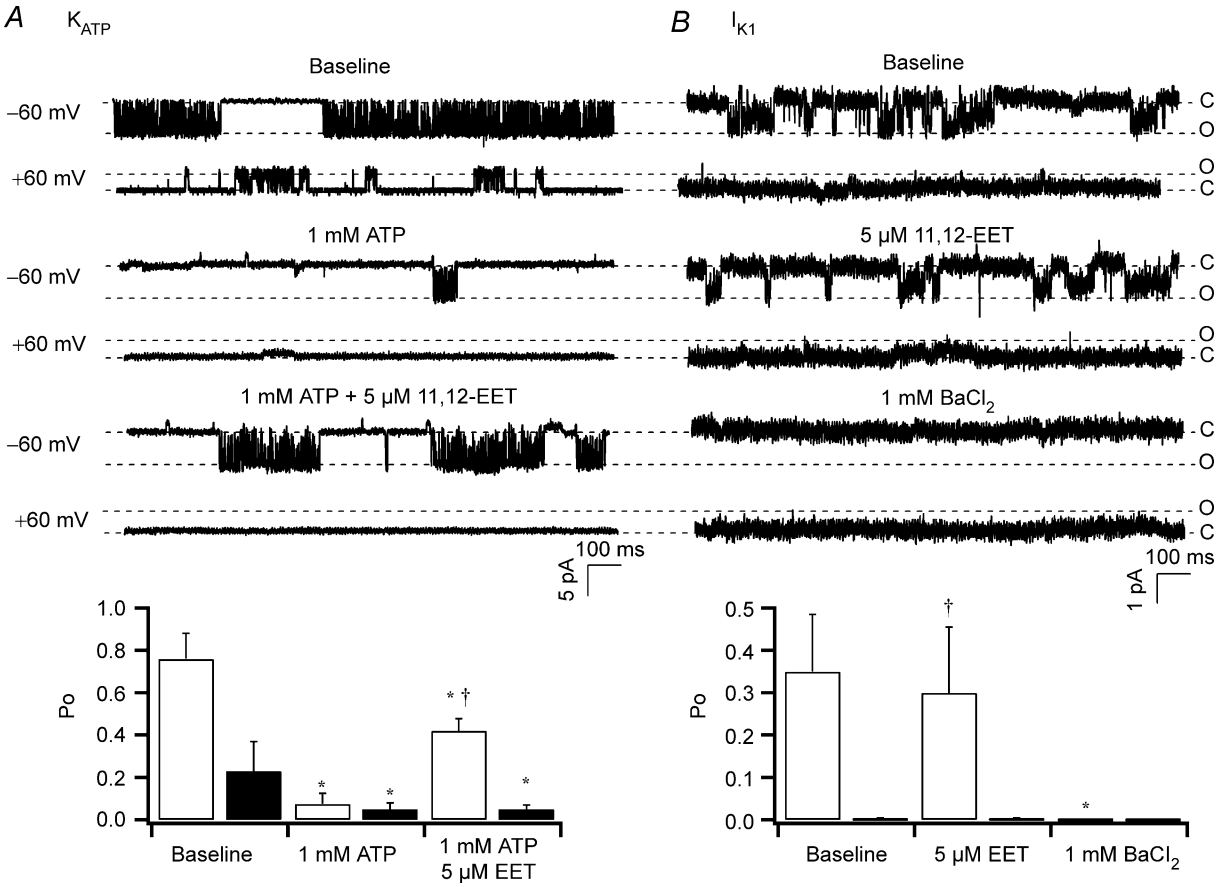

Figure 3. Effects of 11,12-EET on cardiac K_{ATP} and I_{K1} channels in rat ventricular myocytes
A, inside-out single cardiac K_{ATP} currents were elicited from −60 mV and +60 mV in symmetrical K^+ (140 mM) at baseline (0 mM ATP), on exposure to 1 mM ATP, and 1 mM ATP plus 5 μM 11,12-EET. Cardiac K_{ATP} channel activities were robustly activated by 11,12-EET even in the presence of 1 mM ATP. *B*, inside-out single I_{K1} currents were recorded from −60 mV and +60 mV in symmetrical K^+ (140 mM) at baseline, on exposure to 5 μM 11,12-EET, and 1 mM $BaCl_2$. 11,12-EET had no effect on I_{K1} channel activities, which were totally inhibited by 1 mM $BaCl_2$. Dashed lines indicate the levels of channel open state (o) and closed state (c). Bar graphs (lower panels) summarizing the effects of 5 μM 11,12-EET on P_o of K_{ATP} and I_{K1} channels. Open bars represent the P_o at −60 mV and filled bars the P_o at +60 mV. $^*P < 0.05$ *versus* baseline. †$P < 0.05$ *versus* 1 mM ATP or 1 mM $BaCl_2$. $n = 3$ for each group.

K_{ATP} currents in WT mice were -4.85 ± 1.03 pA pF^{-1} (HP = 0 mV, TP = − 100 mV, $n = 5$), but those in the CYP2J2 transgenic mice were 190% higher at -14.01 ± 4.84 pA pF^{-1} ($n = 6$, $P < 0.05$ *versus* WT). Exposure to 1 μM AA increased the K_{ATP} currents in WT to -11.66 ± 2.60 pA pF^{-1} ($n = 5$, $P < 0.05$ *versus* baseline) and produced a further substantial increase in CYP transgenic mice to -30.96 ± 11.34 pA pF^{-1} ($n = 6$, $P < 0.05$ *versus* baseline, and *versus* WT with AA). The cardiac K_{ATP} channel *I–V* relationships of WT and CYP2J2 transgenic mice are shown in Fig. 5*C*. Similar to that observed in rats, AA only enhanced the inward components of cardiac K_{ATP} currents in mice.

To determine whether K_{ATP} channels are up-regulated in the CYP2J2 transgenic hearts, we determined the expression of channel proteins, Kir6.2 and SUR2A, by Western blotting. There was no significant difference in Kir6.2 and SUR2A expression between CYP2JA transgenic and WT hearts (Fig. 6*A* and *B*). Hence, the higher K_{ATP} current densities in the CYP2J2 transgenic myocardium are likely to represent more robust K_{ATP} channel activation at baseline. Exposure to AA further enhanced the K_{ATP} currents, suggesting that CYP activity might be a rate-limiting step.

Immunoblotting with two different CYP2J2 antibodies revealed that CYP2J2 transgenic mice had abundant cardiac-specific expression of the transgene, whereas WT littermates did not (Fig. 6*C*). Densitometry of the immunoblots performed with anti-CYP2J2rec antibody revealed an approximately 2-fold increase in CYP2J protein expression in CYP2J2 transgenic *versus* WT hearts. Previous work has confirmed that microsomes from CYP2J2 transgenic hearts had approximately 3-fold higher AA epoxygenase activity than microsomes from WT hearts (Seubert *et al.* 2004). Moreover, CYP2J2 transgenic cardiac myocytes released significantly more stable EET

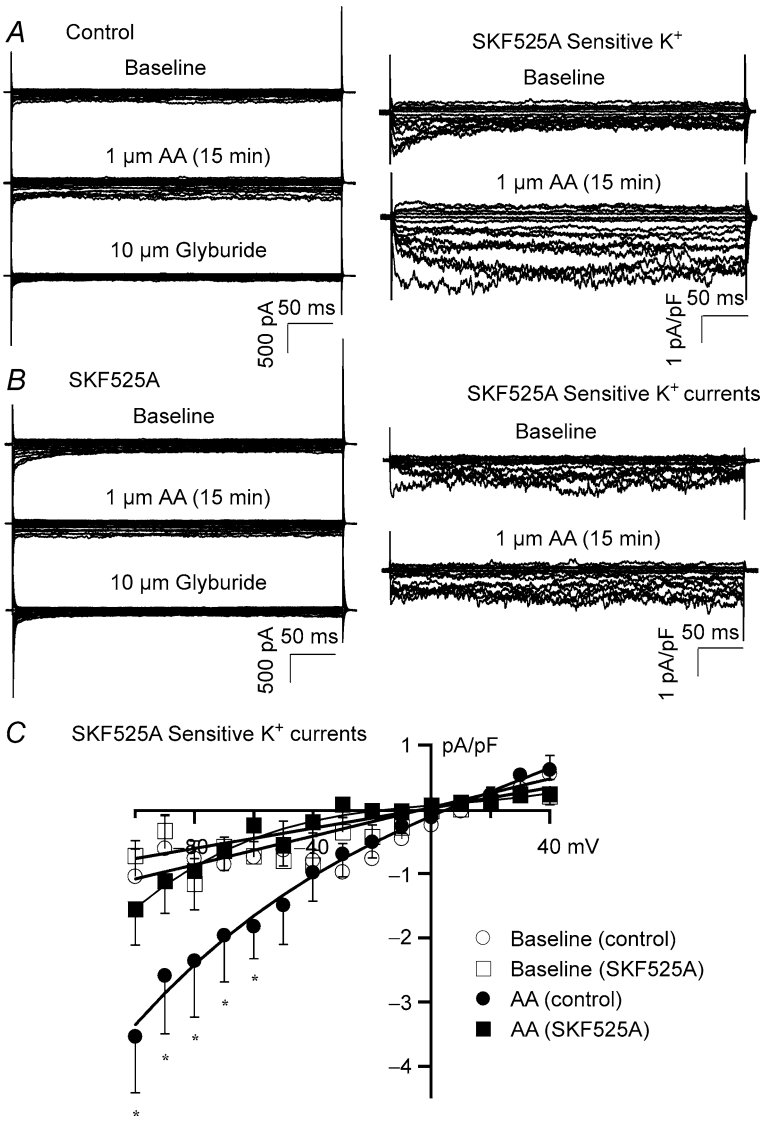

Figure 4. Effects of AA metabolites on K_{ATP} channel activities in rat mesenteric arterial smooth muscle cells
A, whole-cell total K^+ currents in rat mesenteric arterial smooth muscle cells were elicited from −100 mV to + 40 mV at a holding potential of 0 mV at 10 mV increments under control conditions. Total K^+ currents were enhanced by 1 μM AA and blocked by 10 μM glyburide (left column). Glyburide-sensitive K_{ATP} currents were obtained by digital subtraction of glyburide-insensitive components of K^+ currents. AA (1 μM) increased vascular K_{ATP} currents (right column). *B*, whole cell total K^+ currents were recorded from cells preincubated with 5 μM SKF525A for 60 min. AA failed to activate total K^+ currents (left column). AA had no effect on vascular K_{ATP} currents (right column). *C*, vascular K_{ATP} channel *I–V* relationships showing the current densities before and after exposure to AA, with and without preincubation with SKF525A. $n = 6$ for all groups; $^*P < 0.05$, AA *versus* baseline.

metabolites into culture medium than did WT cardiac myocytes (Seubert *et al.* 2004).

To determine whether the higher baseline level of cardiac K_{ATP} currents in CYP2J2 transgenic mice is due to enhanced CYP activities, we inhibited CYP activities with miconazole. Incubation with 10 μM miconazole for 60 min not only abolished the AA effects on K_{ATP} channel activation in both WT and CYP2J2 transgenic mice (Fig. 7*A*), but also reduced the current densities in CYP2J2 transgenic mice to those of WT mice (-0.75 ± 0.56 pA pF^{-1} in WT *versus* -1.03 ± 0.32 pA pF^{-1} in transgenic mice, $n = 6$ for both, N.S., Fig. 7*B*). K_{ATP} channel *I–V* relationships of WT and CYP2J2 transgenic mice pretreated with miconazole, before and after exposure to AA are shown in Fig. 7*C*. These results indicate that enhanced baseline K_{ATP} channel activities in CYP2J2 transgenic mice are due to the increased formation of CYP epoxygenase metabolites.

Activation of K_{ATP} channels by EETs is mediated through different mechanisms in rat ventricular myocytes and arterial smooth muscle cells

To further determine the effects of EETs on K_{ATP} channel activation in cardiac myocytes and vascular smooth muscle cells, we compared the effects of intracellular and extracellular applications of 11,12-EET. In rat cardiac myocytes, 5 μM 11,12-EET applied extracellularly (in the bath solution) increased K_{ATP} currents from -2.17 ± 0.83 pA pF^{-1} to -4.53 ± 0.56 pA pF^{-1} (HP = 0 mV, TP = -100 mV, $n = 6$, $P < 0.05$), and from -0.32 ± 0.21 pA pF^{-1} to -0.53 ± 0.16 pA pF^{-1} (TP = -30 mV, $n = 6$, N.S.) (Fig. 8*A*, left column). In contrast, 5 μM 11,12-EET applied intracellularly (in the pipette solution) enhanced K_{ATP} currents by 850% from -1.37 ± 0.80 pA pF^{-1} to -12.46 ± 3.98 pA pF^{-1} (TP = -100 mV, $n = 6$, $P < 0.05$

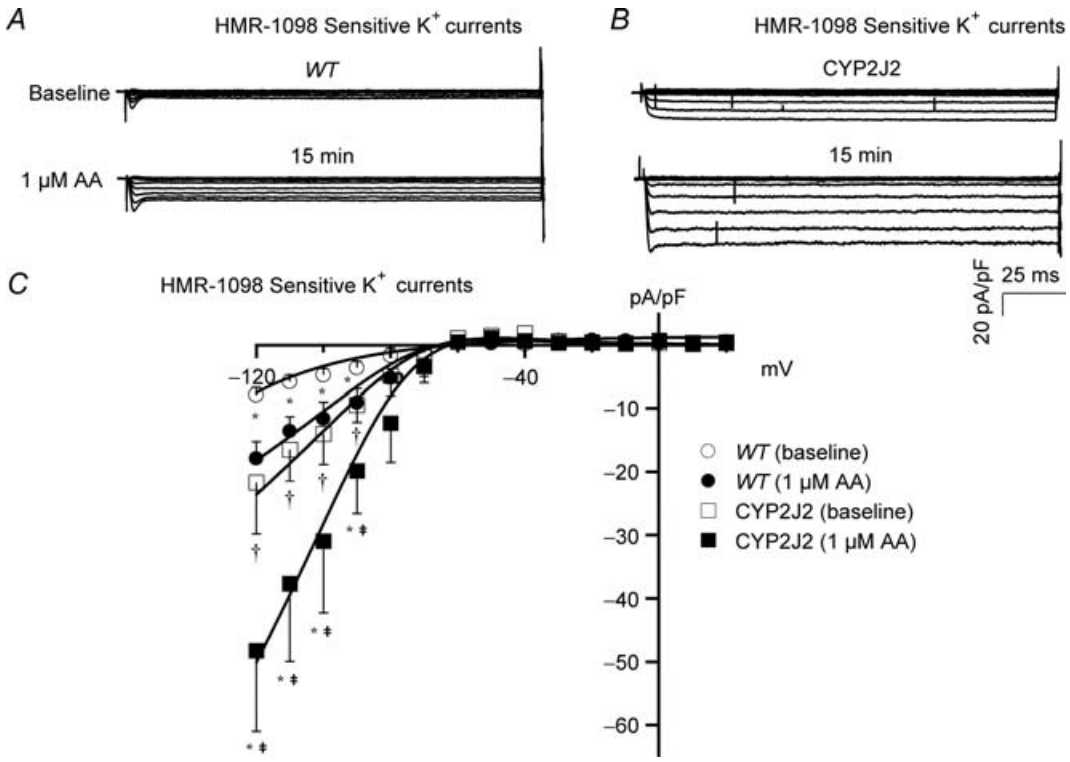

Figure 5. Effects of AA metabolites on cardiac K_{ATP} channel activities in transgenic mice overexpressing CYP2J2

Representative tracings of cardiac K_{ATP} currents (after subtraction of HMR-1098-insensitive K^+ currents) were recorded from ventricular myocytes of WT mice (*A*) and transgenic mice overexpressing CYP2J2 (*B*), before (upper panels) and 15 min after (lower panels) the application of AA (1 μM). Baseline K_{ATP} current densities were 2-fold higher in the CYP2J2 transgenic mice than in WT. Exposure to 1 μM AA enhanced the K_{ATP} current densities in both WT and transgenic mice. *C*, K_{ATP} channel *I–V* relationships showing significant increase in current densities at voltages negative to E_K on exposure to AA in both WT and CYP2J2 transgenic mice. *$P < 0.05$ AA *versus* baseline for both WT and CYP2J2 transgenic mice. $n = 5$ for WT and $n = 6$ for CYP2J2 transgenic mice; †$P < 0.05$ baseline of CYP2J2 *versus* baseline of WT; ‡$P < 0.05$ CYP2J2 transgenic with AA *versus* WT with AA.

versus baseline and *versus* EET applied extracellularly), and -0.20 ± 0.14 pA pF^{-1} to -0.53 ± 0.16 pA pF^{-1} (TP $= -30$ mV, $n = 6$, N.S. *versus* baseline and EET applied extracellularly) (Fig. 8*A*, right column). Hence, similar to AA, 11,12-EET only enhanced the inward currents of cardiac K_{ATP} channels. The effects of 11,12-EET reached a steady-state about 10 min after the whole-cell configuration was established with membrane break-through. This may reflect the time course of buffer equilibration between the pipette solution and the intracellular milieu.

In rat mesenteric arterial smooth muscle cells, 5 μM 11,12-EET applied extracellularly markedly increased K_{ATP} currents from -2.00 ± 0.54 pA pF^{-1} to -11.10 ± 2.08 pA pF^{-1} (HP $= 0$ mV, TP $= -100$ mV, $n = 8$, $P < 0.05$) (Fig. 8*B*). In contrast, 5 μM 11,12-EET applied intracellularly had no effects (from -1.11 ± 0.14 pA pF^{-1} to -1.78 ± 1.04 pA pF^{-1}, $n = 4$, N.S.) (Fig. 8*B*). The *I–V* relationships of cardiac and vascular K_{ATP} channels are shown in Fig. 8*C* and *D*, respectively. Unlike cardiac K_{ATP} channels, which were activated by 11,12-EET only at voltages negative to E_K, vascular K_{ATP} channels were enhanced by extracellular 11,12-EET at almost all voltages including both the inward and outward components. These results suggest that activation of cardiac and vascular K_{ATP} channels by EETs is mediated through different mechanisms.

Role of PKA in the activation of cardiac and vascular K_{ATP} channels by EET

It is unknown whether cAMP-dependent signalling is involved in the activation of cardiac K_{ATP} channels by EETs. We examined the role of PKA on the activation of cardiac and vascular K_{ATP} channels by EETs by treating the cells for 60 min with 5 μM myristoylated PKI amide, a membrane-permeant PKA

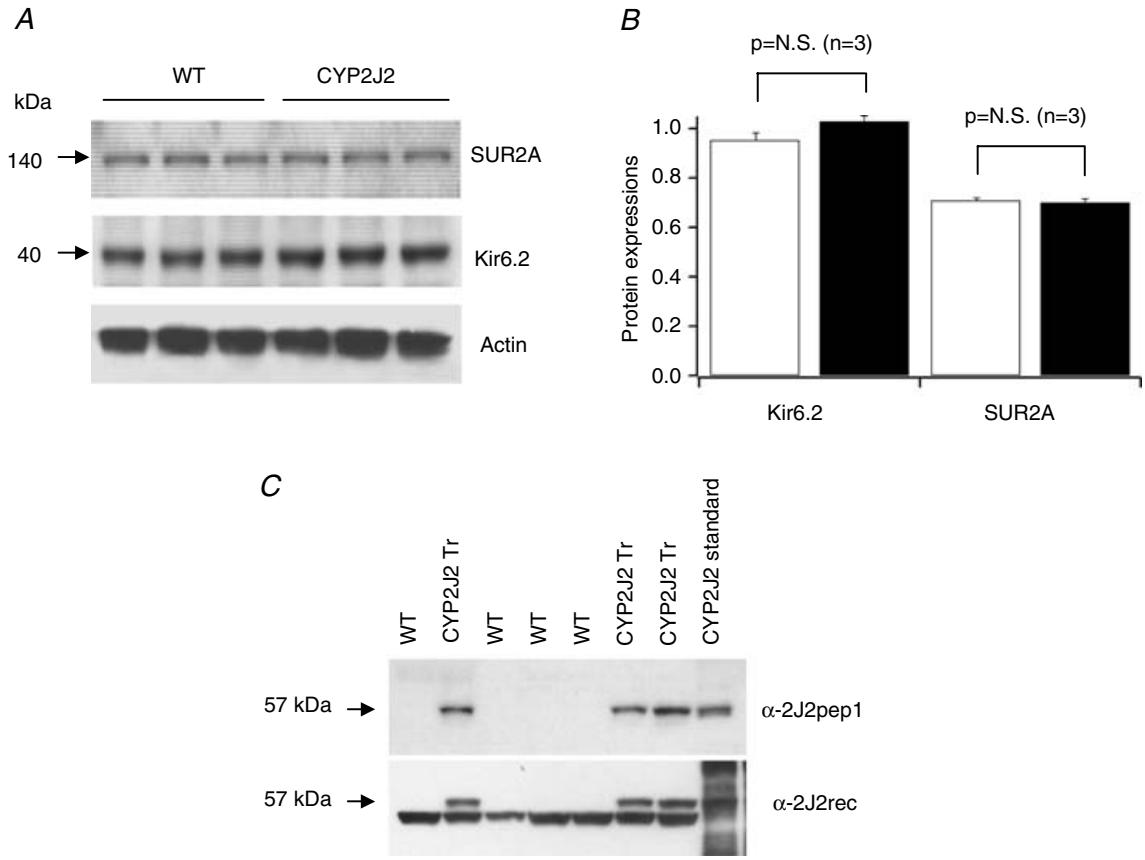

Figure 6. Immunoblot analysis of Kir6.2, SUR2A, and CYP2Js expression in hearts of WT and CYP2J2 transgenic mice

A, Western blot showing that the expression of Kir6.2 and SUR2A is similar between WT and CYP2J2 transgenic hearts. Actin serves as the loading control. *B*, group data from three pairs of WT and CYP2J2 transgenic mice. There is no difference in SUR2A and Kir6.2 protein expression between WT and CYP2J2 transgenic hearts. *C*, Western blots showing that expression of CYP2J2 in cardiac myocytes of WT and CYP2J2 transgenic mice. Anti-CYP2J2pep1 is specific for CYP2J2 and anti-CYP2J2rec is a polyclonal antibody against human recombinant CYP2J2 and cross reacts with murine cardiac CYP2J.

inhibitor. After PKI treatment in rat ventricular myocytes, 5 μM 11,12-EET applied extracellularly had a very small effect on the K_{ATP} currents, from -1.41 ± 0.40 pA pF^{-1} to -2.86 ± 0.67 pA pF^{-1} (HP = 0 mV, TP = -100 mV, $n = 5$, $P < 0.05$) (Fig. 9*A*). However, 5 μM 11,12-EET applied intracellularly produced a marked increase in activation of K_{ATP} currents, from -1.76 ± 0.67 pA pF^{-1} at baseline to -11.46 ± 4.50 pA pF^{-1} ($n = 5$, $P < 0.05$ *versus* baseline, and *versus* EET applied extracellularly) (Fig. 9*B*). Hence, treatment with PKI did not alter the activation of cardiac K_{ATP} channel by 11,12-EET, indicating that the effects of EET were not mediated through PKA-dependent mechanisms. However, the baseline cardiac K_{ATP} currents were significantly reduced by PKI, suggesting that PKA may modulate the basal activities of the channel. Figure 9*C* shows the K_{ATP} *I–V* relationships for these studies.

In contrast, after PKI treatment in mesenteric arterial smooth muscle cells, the effects of extracellularly applied 5 μM 11,12-EET on the K_{ATP} currents were significantly reduced (TP = -100 mV, -1.28 ± 0.42 pA pF^{-1} at baseline to -3.94 ± 0.52 pA pF^{-1}, $n = 5$, $P < 0.05$), producing only 34% of the effects in control (Fig. 9*D*). The channel *I–V* relationships are shown in Fig. 9*E*. These results suggest that the PKA signalling pathway plays an important role in the activation of K_{ATP} channels by EETs in vascular smooth muscle cells. Comparison between the effects of 11,12-EET on activations of cardiac and vascular K_{ATP} channels, in the presence and absence of PKI are summarized in Fig. 9*F*.

Since the activation of vascular BK channels by EETs involves ADP-ribosylation of Gsα (Li *et al.* 1999), we tried to further delineate the signalling mechanism by examining the effects of 11,12-EET on the activation of cardiac and vascular K_{ATP} channels after treatment with 100 μM *m*-iodobenzylguanidine (MIBG), an ADP-ribosyltransferase inhibitor. In rat ventricular myocytes after treatment with MIBG, exposure to 5 μM 11,12-EET in the bath solution produced a 234% increase in K_{ATP} currents, from a baseline of -0.99 ± 0.33 pA pF^{-1} to -3.34 ± 1.02 pA pF^{-1} (TP = -100 mV, $n = 6$, $P < 0.05$) (Fig. 10*A*). 11,12-EET (5 μM) applied intracellularly produced a 689% increase in K_{ATP} currents, from -1.07 ± 0.70 pA pF^{-1} to -7.34 ± 1.82 pA pF^{-1} ($n = 6$, $P < 0.05$ *versus* baseline, and *versus* EET applied

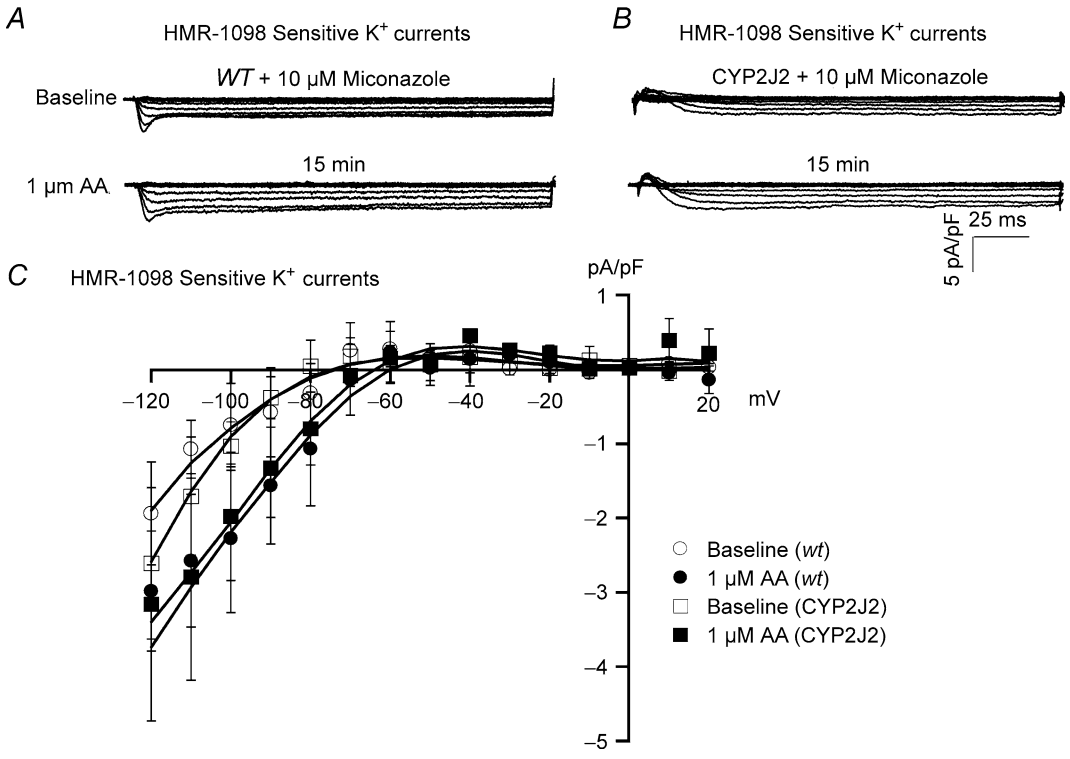

Figure 7. Effects of AA on cardiac K_{ATP} channel activities are abolished by treatment with miconazole
HMR-1098-sensitive K_{ATP} currents were recorded in ventricular myocytes from WT (*A*) and CYP2J2 transgenic mice (*B*) preincubated with 10 μM miconazole (60 min), before (upper panels) and 15 min after (lower panels) the application of 1 μM AA. Treatment with miconazole not only abolished the effects of AA on K_{ATP} channel activities in both WT and CYP2J2 transgenic mice, but also reduced the baseline current densities of the CYP2J2 transgenic myocytes to that of WT. *C*, after treatment with miconazole, K_{ATP} channel *I–V* relationships showed no significant difference in K_{ATP} channel activities between WT and CYP2J2 transgenic cardiac myocytes before and after exposure to AA. $n = 6$ for both groups.

extracellularly) (Fig. 10*B*). Figure 10*C* shows the cardiac K_{ATP} channel *I–V* relationships before and after 5 μM 11,12-EET applied intracellularly or extracellularly in the presence of MIBG. These results were similar to those obtained without MIBG incubation, suggesting that the EET effects were independent of ADP-ribosylation.

In contrast, incubation with MIBG totally abolished the effects of 11,12-EET on vascular K_{ATP} channel activation (TP $= -100$ mV, -1.73 ± 0.51 pA pF^{-1} at baseline and -2.50 ± 1.02 with 11,12-EET, $n = 6$, N.S.) (Fig. 10*D*), suggesting that ADP-ribosyltransferase activity is crucial in mediating the activation of vascular K_{ATP} channels by EETs. Figure 10*E* shows the *I–V* relationships and illustrates that 11,12-EET applied extracellularly had no effect on vascular K_{ATP} channel activities at all voltages examined. We did not examine the effects of intracellularly applied 11,12-EET after MIBG incubation since there was no vascular K_{ATP} channel activation by EETs at baseline (see the right column of Fig. 8*B*). Comparison between the effects of 11,12-EET on cardiac and vascular K_{ATP} channels, with and without MIBG incubation are summarized in Fig. 10*F*.

Discussion

We have made a number of key observations in this study. First, we have demonstrated that AA activates cardiac and vascular K_{ATP} channels through CYP metabolites. Second, EETs activate cardiac and vascular K_{ATP} channels through different mechanisms. Specifically, EETs potently activated cardiac K_{ATP} channels when applied intracellularly but activated vascular K_{ATP} channels when applied extracellularly. Third, activation of cardiac K_{ATP} channels by EETs does not involve c-AMP dependent signalling, whereas activation of vascular K_{ATP} channels by EETs is dependent on PKA and ADP-ribosyltransferase activities. Fourth, cardiac K_{ATP} channel activities in rat and mice are tightly coupled to CYP epoxygenase activities.

EETs are endothelium-derived hyperpolarizing factors (EDHFs) that modulate vascular tone in various vessel

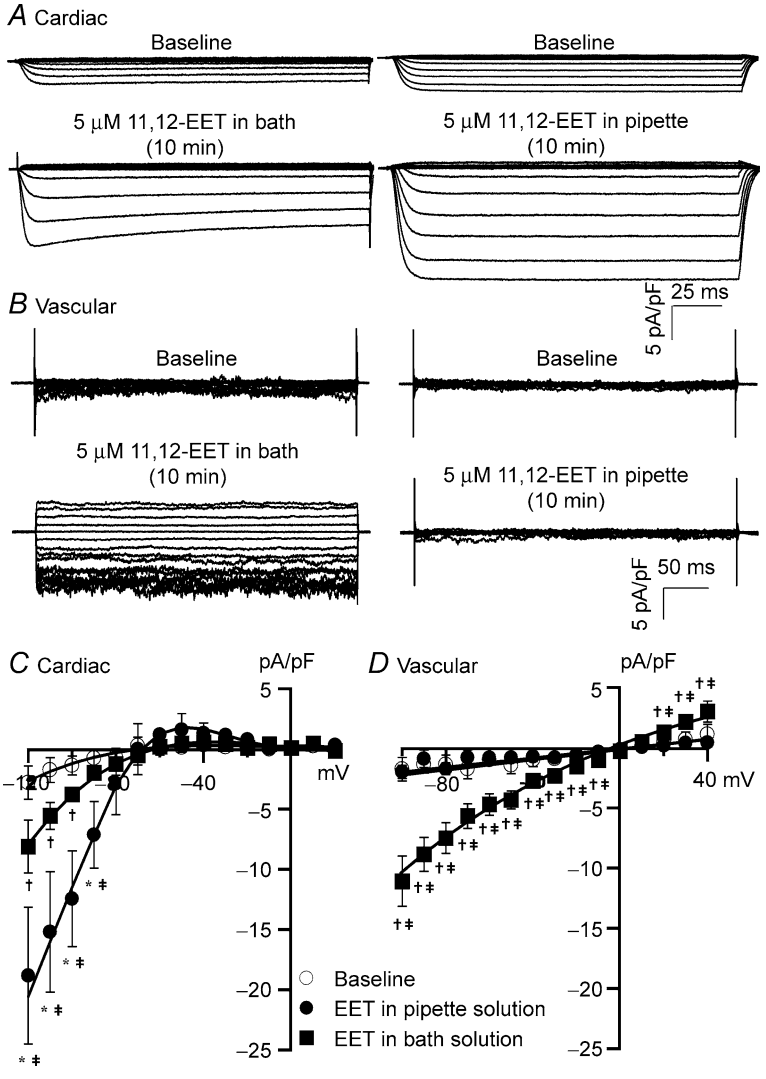

Figure 8. Cardiac and vascular K_{ATP} channels are activated by 11,12-EET through different mechanisms
A, recordings of whole-cell K_{ATP} currents (after subtraction of glyburide-insensitive K^+ currents) from rat ventricular myocytes. Extracellularly applied 11,12-EET (5 μM) produced small enhancement of K_{ATP} currents after 10 min of exposure (left panel), while intracellularly applied 11,12-EET resulted in a much greater stimulation of current activities (right panel). Cardiac K_{ATP} currents were recorded in the presence of 140 mM intracellular K^+ and 4.5 mM extracellular K^+. *B*, vascular K_{ATP} currents recorded from rat mesentery arterial smooth muscle cells. Extracellularly applied 11,12-EET (5 μM) produced significant K_{ATP} current activation (left panel), while intracellularly applied 11,12-EET had no effect (right panel). Vascular K_{ATP} currents were recorded in the presence of symmetrical K^+ concentration at 140 mM. *I–V* relationships of cardiac (*C*) ($n = 6$ for all groups) and vascular K_{ATP} channels (*D*) ($n = 4$ for intracellularly applied 11,12-EET and $n = 8$ for extracellularly applied 11,12-EET), showing the effects of 5 μM 11,12-EET applied intracellularly or extracellularly. $^*P < 0.05$, intracellularly applied 11,12-EET *versus* baseline. †$P < 0.05$, extracellularly applied 11,12-EET *versus* baseline. ‡$P < 0.05$, intracellularly applied *versus* extracellularly applied 11,12-EET.

beds including human coronary arteries (Fisslthaler *et al.* 1999; Archer *et al.* 2003). Inhibition of CYP epoxygenases attenuates EDHF-mediated vasodilatation in resistance arteries (Bolz *et al.* 2000). Products of CYP epoxygenases are among the most potent activators of the large conductance Ca^{2+}-activated K^+ (BK) channels, producing vasodilatation through hyperpolarization of membrane potentials in vascular smooth muscle cells (Lu *et al.* 2001*b*; Ye *et al.* 2002; Weston *et al.* 2005). In isolated rat hearts, application of 5 μM 11,12-EET significantly improved postischaemic cardiac contractility (Wu *et al.* 1997). Transgenic mice overexpressing cardiac CYP2J2 exhibit an improvement in the postischaemic recovery of left ventricular function (Seubert *et al.* 2004). The importance of these findings is underscored by a recent report that a polymorphism in the proximal promoter of human CYP2J2 leads to reduced gene expression and is associated with an increase risk of coronary artery disease, indicating a cardioprotective role of CYP2J2 in humans (Spiecker *et al.* 2004).

K_{ATP} channels are activated by various lipid metabolites including the long chain acyl-CoA esters (Schulze *et al.* 2003), phosphatidylinositol 4,5-bisphosphate (Baukrowitz *et al.* 1998; Shyng & Nichols, 1998) and L-palmitoylcarnitine (Haruna *et al.* 2000). We have previously reported that action potentials in cardiac myocytes can be modulated by EETs through activation of K_{ATP} channels. The effects of EET on cardiac K_{ATP} channels have an EC_{50} of 30 nM and do not require the presence of intracellular signalling agents such as GTP (Lu *et al.* 2001*a*). Unlike other lipid metabolites, the effects of EET on cardiac K_{ATP} channels are stereospecific (Lu *et al.* 2002). Using site-directed mutagenesis, we have determined that R192 and R195 on the C-terminus of Kir6.2 are critical sites for the EET effects (Lu *et al.* 2005). However, the physiological relevance of this finding is still

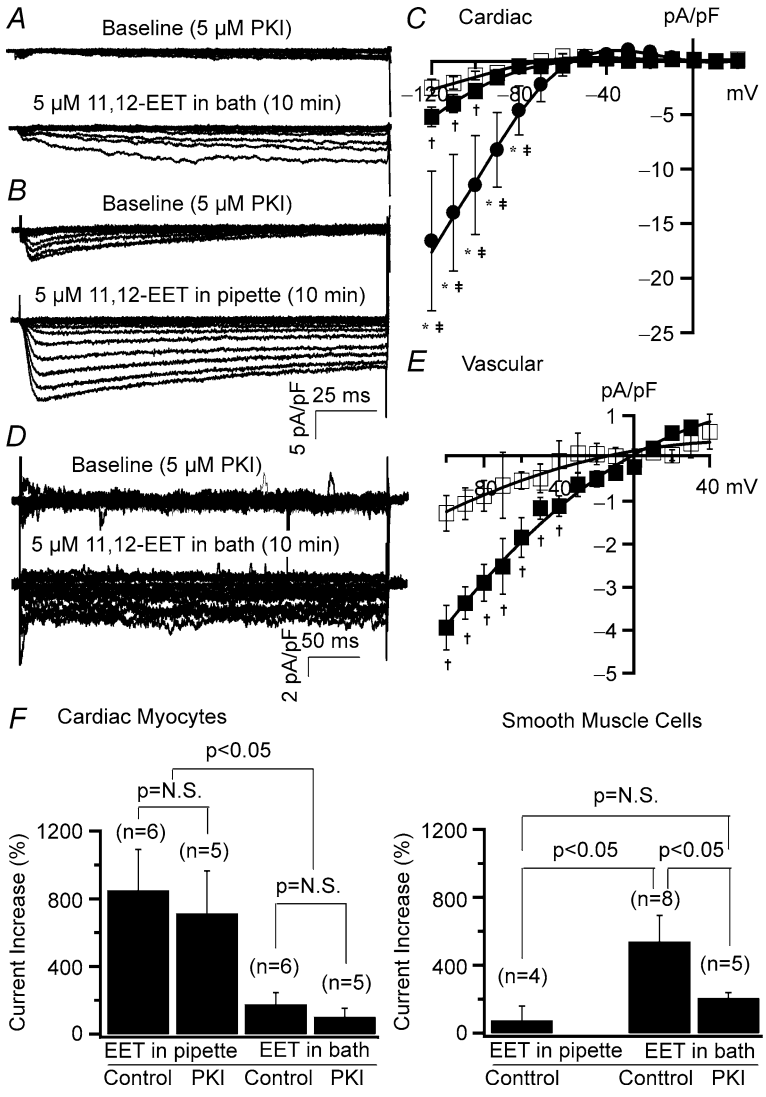

Figure 9. Effects of 11,12-EET on cardiac K_{ATP} channels are not inhibited by PKI
Recordings of HMR-1098-sensitive cardiac K_{ATP} currents in rat cardiac myocytes after treatment with 5 μM myristoylated PKI amide, showing the effects of extracellularly applied (*A*) and intracellular applied (*B*) 11,12-EET (5 μM). Pretreatment with myristoylated PKI amide had no effects on cardiac K_{ATP} channel sensitivity to 11,12-EET. *C*, cardiac K_{ATP} channel *I–V* relationships with no EET treatment (○), and with 11,12-EET applied extracellularly (■) or intracellularly (●). $n = 5$ for all groups; $^*P < 0.05$, intracellularly applied 11,12-EET *versus* baseline; †$P < 0.05$, extracellular 11,12-EET *versus* baseline; ‡$P < 0.05$, intracellular application *versus* extracellular application. *D*, recordings of glyburide-sensitive vascular K_{ATP} currents in rat mesenteric smooth muscle cells after treatment with 5 μM myristoylated PKI amide showing that the effects of extracellularly applied 11,12-EET were significantly attenuated. *E*, vascular K_{ATP} channel *I–V* relationship after treatment with PKI at baseline (□) and after exposure to 11,12-EET (■). The effects of 11,12-EET were significantly inhibited by PKI (compare with Fig. 8*D*). $n = 5$; $^*P < 0.05$, effect of 11,12-EET *versus* baseline. *F*, bar graphs comparing the percentage K_{ATP} current increase in cardiac myocytes (left panel) and vascular smooth muscle cells (right panel) by 11,12-EET applied intracellularly and extracellularly with and without (control) treatment with PKI. Group data showing the cardiac and vascular K_{ATP} current densities measured at −100 mV. Incubation with PKI had no effects on the activation of cardiac K_{ATP} channels by 11,12-EET, but significantly inhibited that in vascular K_{ATP} channels.

not fully understood. In particular, the ability of endogenously produced EETs to activate cardiac K_{ATP} channels in the presence of physiological concentrations of cytoplasmic ATP has not been previously demonstrated. The results of the present study show that CYP metabolites of AA could enhance cardiac K_{ATP} currents severalfold. Application of 1 μM AA produced a 219% increase in K_{ATP} channel activities. The AA effects were mediated by CYP epoxygenase products, because they could be completely abolished by CYP epoxygenase inhibitors, miconazole or SKF525A. These inhibitors also reduced the baseline K_{ATP} currents suggesting that the steady-state production of EETs in cardiac myocytes is important in regulating the basal K_{ATP} currents in these cells. In CYP2J2 transgenic mice, cardiac K_{ATP} current densities were 190% higher than that of WT, suggesting that basal K_{ATP} channel activities are closely regulated by CYP2J2 products. Moreover, exposure to 1 μM AA resulted in further stimulation of K_{ATP} channel activities to levels that were 520% higher than that of WT at baseline. The enhanced K_{ATP} channel activities in the transgenic animals are not due to increased channel expression but are the result of enhanced CYP activities since miconazole reduced channel activities to that of WT and abolished the AA effects. Our results from the CYP2J2 transgenic animals have important physiological and clinical implications since CYP enzymes are known to be induced by chemicals and drugs, some of which are commonly used in clinical practice (Fisslthaler *et al.* 2003). Hence, AA can modulate the cardiac action potential through activation of K_{ATP} channels by its CYP metabolites.

Another important observation is that the effects of CYP products of AA, mainly EETs, on cardiac K_{ATP} channels are voltage dependent, which is consistent

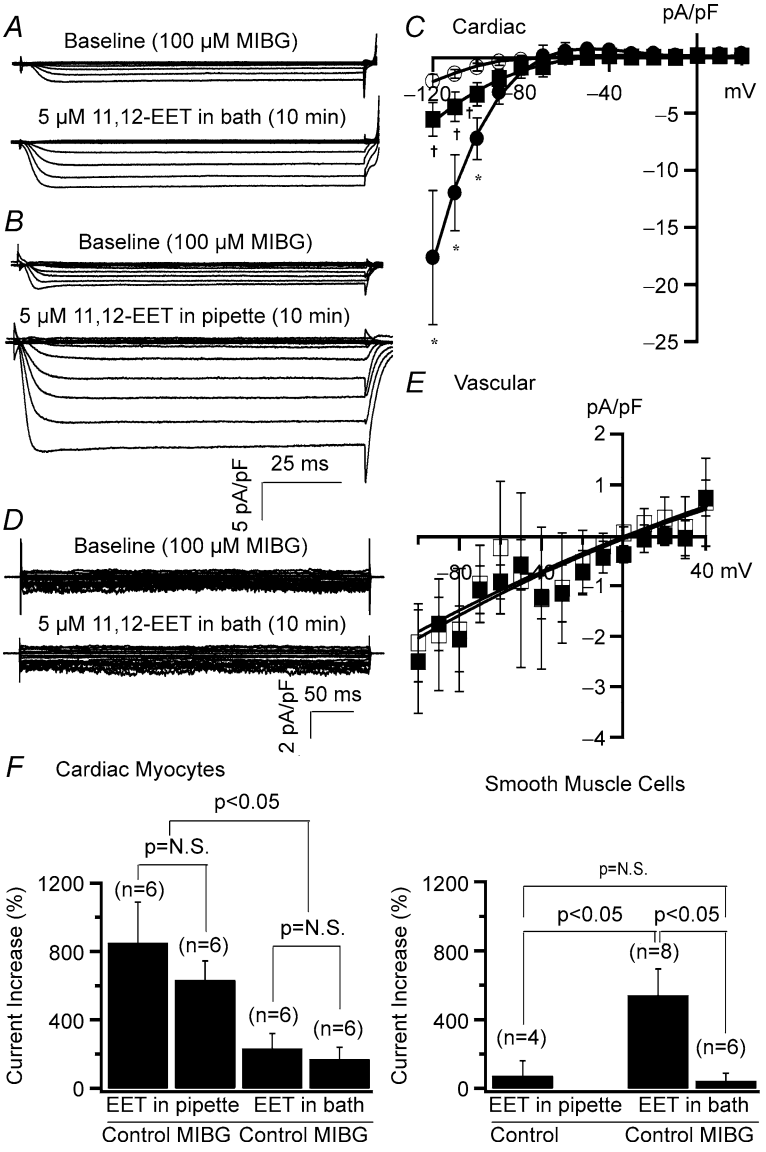

Figure 10. Effects of 11,12-EET on cardiac K_{ATP} channels are not inhibited by MIBG
Recordings of HMR-1098-sensitive K_{ATP} currents recorded in rat ventricular myocytes after incubation with 100 μM MIBG (60 min), showing the effects of extracellularly (*A*) and intracellularly (*B*) applied 11,12-EET (5 μM). Incubation with MIBG (60 min) did not alter the activation of cardiac K_{ATP} channels by 11,12-EET. *C*, cardiac K_{ATP} channel *I–V* relationships with no EET treatment (○), and with 11,12-EET (5 μM) applied extracellularly (■) or intracellularly (●). $n = 6$ for all groups; $^*P < 0.05$, intracellularly applied 11,12-EET *versus* baseline; †$P < 0.05$, extracellularly applied 11,12-EET *versus* baseline; ‡$P < 0.05$, intracellularly applied *versus* extracellularly applied 11,12-EET. *D*, recordings of glyburide-sensitive K_{ATP} currents in rat mesenteric smooth muscle cells after treatment with 100 μM MIBG. *E*, vascular K_{ATP} channel *I–V* relationships after treatment with MIBG at baseline (□) and after exposure to 11,12-EET (■), showing that the effects of extracellularly applied 11,12-EET were abolished, $n = 6$. *F*, bar graphs comparing the percentage K_{ATP} current increase in cardiac myocytes (left panel) and vascular smooth muscle cells (right panel) by 11,12-EET applied intracellularly and extracellularly with and without treatment with MIBG. Group data showing the cardiac and vascular K_{ATP} current densities measured at −100 mV.

with our previous observation in single channel studies (Lu *et al.* 2001*a*). Cardiac K_{ATP} currents were activated preferentially at voltages negative to E_K, hence potentiating the inward rectifying properties of the channel, converting it from a weak into a relatively strong inward rectifier. This has important physiological and clinical implications because a strong inward rectifier K^+ current is potentially antiarrhythmic, maintaining a hyperpolarized membrane potential and preventing the development of automaticity (Dhamoon & Jalife, 2005). Indeed, transgenic animals with ablation of the Kir6.2 gene are predisposed to catecholamine-induced ventricular arrhythmia (Liu *et al.* 2004). We have also found that 11,12-EET had no effect on the activities of cardiac I_{K1}, the major inward rectifier K^+ channel in the heart (Fig. 3). Hence, during cardiac ischaemia when EET production is enhanced (Nithipatikom *et al.* 2001) and I_{K1} activities are reduced (Aimond *et al.* 1999), activation of K_{ATP} channels by EETs may provide antiarrhythmic protection in addition to the cardioprotection effects against reperfusion injuries (Nithipatikom *et al.* 2006). EETs are unique cardiac K_{ATP} channel activators because of the voltage dependence of channel activation (Figs 1, 2 and 3) and the stereo-specific effects (Lu *et al.* 2002). These unusual properties could potentially be exploited for therapeutic strategies. Currently, the mechanism of voltage dependence of EET effects on cardiac K_{ATP} channels is not fully understood. Whether the modulation of K_{ATP} currents by EETs plays a role in mediating cardioprotection from antiarrhythmic effects deserves further investigation.

In addition to the direct effects on ion channels, EETs are known to activate various kinase signalling pathways, including tyrosine kinases, MAP kinases, extracellular signal regulated kinases, as well as protein kinase B/Akt (Fleming *et al.* 2001), and have been shown to modulate ion channels through second messenger signalling pathways. Enhancement of bovine coronary BK channel activities by EETs is mediated through ADP-ribosylation of Gsα (Li *et al.* 1999). EETs have been shown to activate rat cardiac L-type Ca^{2+} currents through increased intracellular cAMP (Xiao *et al.* 1998, 2004). The MAP kinase signalling pathway has been shown to mediate mitochondria K_{ATP} channel activation by EETs (Seubert *et al.* 2004). Recently, we have reported that activation of mesenteric smooth muscle K_{ATP} channels by EETs is dependent on PKA activities and this contributes to 50% of the total vasodilatation by EETs in these vessels (Ye *et al.* 2005, 2006). However, the molecular steps through which EETs activate vascular K_{ATP} channels are still unclear. Specific cell surface EET 'receptors' have been postulated to exist. The specific high affinity binding sites for 14,15-EET in U-937 cells may be associated with an EET receptor (Wong *et al.* 1997). The regio- and enantio-specific effects of EETs on vasoreactivity and ionic channel regulation strongly suggests that they may involve a 'receptor' mechanism. In renal arterial smooth cells, only the 11(R),12(S)-EET enantiomer, but not the 11(S),12(R)-EET enantiomer nor the 5,6–8,9- and 14,15-EET regioisomers, could activate BK currents (Zou *et al.* 1996). In rat cortical collecting ducts, only 11,12-EET could inhibit epithelial Na^+ channel activity, while the other EET regioisomers had no effects (Wei *et al.* 2004). In bovine coronary arterial smooth muscle cells, pretreatment with 14,15-EEZE-mSI preferentially inhibited the 14,15-EET effects on BK channel activation and vasodilatation (Gauthier *et al.* 2003), suggesting specific structural requirements of the activities of EETs. Recently, cAMP-induced aromatase activities in cultured aortic smooth muscle cells were found to be inhibited by 14,15-EET, by the non-metabolized *N*-methylsulfanilamide derivative of 14,15-EET (14,15-EET-SI), and by the membrane-impermeant 14,15-EET-SI that was covalently tethered to silica beads. These results suggest the presence of a specific EET binding site, possibly a receptor, in the plasma membrane (Snyder *et al.* 2002). Our results on the effects of EETs on vascular K_{ATP} channels are consistent with the presence of a cell surface EET receptor since EETs exert their effects mainly from the extracellular side of the cell membrane. Intracellular application of EETs has minimal effect on vascular K_{ATP} channels and the EET effects were dependent on ADP-ribosyltransferase and PKA activities. However, definitive mechanisms have to await direct identification of the putative EET receptor(s). We have also noted that exposure to AA produced a small but significant increase in the vascular K_{ATP} current at very negative voltages. The mechanism of this increase is not entirely clear at present but could be due to formation of CYP products other than EETs or possible synergistic effects of the EET regioisomers.

In the present study, the contrast between the mechanisms through which EETs activate cardiac and vascular K_{ATP} channels is quite impressive. 11,12-EET activates cardiac K_{ATP} channels most effectively from the cytoplasmic side, whereas activation of the vascular K_{ATP} channels occurs from the extracellular surface of the membrane. In addition, treatments with PKI and MIBG did not alter the sensitivity of K_{ATP} channels to 11,12-EET in cardiac myocytes, but totally abolished it in vascular smooth muscle cells. The mechanisms that underlie such disparity can be partly explained by the inherent structural and pharmacological difference between these channels. First, cardiac K_{ATP} channels are composed of SUR2A and Kir6.2 subunits, and vascular channels of SUR2B and Kir6.1 (Seino & Miki, 2003). Second, cardiac and vascular K_{ATP} channels have different sensitivity to ATP. The ATP inhibition curve of cardiac K_{ATP} channels is sigmoidal with an IC_{50} of 30 μM, while that of vascular K_{ATP} channels is bell-shaped with channel stimulation by 0.1–1 mM ATP

and channel inhibition by 3–5 mM ATP (Yokoshiki *et al.* 1998). Third, the electrophysiological properties of the two channels are different. The cardiac K_{ATP} channel is weakly inwardly rectifying with a conductance of 80 pS in symmetrical K^+, whereas the vascular K_{ATP} channel *I–V* relationship is almost linear with a conductance of 33 pS. Fourth, their pharmacological properties are different. Nucleotide diphosphate (NDP) stimulation by ADP, UDP and GDP is critical for vascular but not cardiac K_{ATP} channel activation (Quayle *et al.* 1997; Yokoshiki *et al.* 1998). Vascular K_{ATP} channels are known to be more sensitive to sulphonylurea drugs such as glyburide and tolbutamide, as well as to the K^+ channel opener diazoxide than cardiac K_{ATP} channels, implying that the properties of the SUR subunit may contribute to the diverse functions of the K_{ATP} channel isoforms. The physiological inference from our results is that K_{ATP} channels in vascular smooth muscle cells can be readily regulated by EETs through paracrine functions, whereas K_{ATP} channels in cardiac myocytes are tightly coupled to endogenous production of EETs which depend on the CYP epoxygenase activities. Since EETs are important signalling agents in the cardiovascular system, regulating a multitude of physiological functions, our findings in this study help delineate the mechanism of K_{ATP} channel regulation by these lipid metabolites and aid our understanding of the role of EETs in the regulation of cardiovascular physiology.

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