

## **Sulfonylurea Receptor-Dependent and -Independent Pathways Mediate Vasodilation Induced by K<sub>ATP</sub> Channel Openers**

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**Running Title:**  $K_{ATP}$  channel opener-induced vasodilation

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**ABBREVIATIONS:** SUR, sulfonylurea receptor; ROS, reactive oxygen species; 4-AP, 4-aminopyridine; MnTMPyP, Manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin; pm $K_{ATP}$ , plasma membrane  $K_{ATP}$ ; mito $K_{ATP}$ , mitochondria  $K_{ATP}$ ; ETC, electron transport chain;  $K_{Ca}$  channels, large-conductance  $Ca^{2+}$ -activated potassium channels;  $K_V$  channels, voltage-gated potassium channels; PSS, physiological saline solution; SOD, superoxide dismutase; RyR channels, ryanodine-sensitive  $Ca^{2+}$  release channels; TTFA, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione; Diaz, diazoxide; Pinac, pinacidil; Ach, acetylcholine; NBD, Nucleotide binding domain; Pecam-1, platelet/endothelial cell adhesion molecule 1; Myh11, myosin, heavy polypeptide 11; PGP9.5, ubiquitin carboxy-terminal hydrolase L1; FABP4, fatty acid binding protein 4.

## Abstract

ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channel openers are vasodilators that activate both plasma membrane and mitochondrial K<sub>ATP</sub> channels. Here, we investigated the molecular mechanisms by which diazoxide and pinacidil induce vasodilation by studying diameter regulation of wild-type (SUR2<sup>+/+</sup>) and sulfonylurea receptor 2 deficient (SUR2<sup>-/-</sup>) mouse myogenic mesenteric arteries. Ryanodine (10 μM), a ryanodine-sensitive Ca<sup>2+</sup> release (RyR) channel blocker, iberiotoxin (100 nM), a large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (K<sub>Ca</sub>) channel blocker, 4-aminopyridine (4-AP, 1 mM), a K<sub>V</sub> channel blocker, MnTMPyP (100 μM), an antioxidant, and a combination of ryanodine and 4-AP reduced diazoxide (100 μM)-induced dilation in pressurized (60 mmHg) SUR2<sup>+/+</sup> arteries by 45-77 %. In contrast, these inhibitors did not alter pinacidil (5 μM)-induced dilation in SUR2<sup>+/+</sup> arteries. RT-PCR indicated that SUR2B was the only SUR isoform expressed in SUR2<sup>+/+</sup> mesenteric artery smooth muscle cells, whereas SURs were absent in SUR2<sup>-/-</sup> cells. In SUR2<sup>-/-</sup> arteries, pinacidil-induced vasodilation was ~10 % of that in SUR2<sup>+/+</sup> arteries, whereas diazoxide-induced vasodilation was similar in SUR2<sup>+/+</sup> and SUR2<sup>-/-</sup> arteries. Atpenin (1 μM), a selective electron transport chain complex II (ETC<sub>II</sub>) inhibitor, dilated arteries similarly to diazoxide and this effect was attenuated by MnTMPyP and ryanodine + 4-AP. Atpenin also attenuated diazoxide-, but not pinacidil-induced vasodilation. In summary, data indicate that pinacidil-induced vasodilation requires SUR2B, whereas diazoxide-induced vasodilation does not require SURs. Rather, diazoxide-induced vasodilation involves ETC<sub>II</sub> inhibition, a smooth muscle cell reactive oxygen species elevation, and RyR, K<sub>Ca</sub>, and K<sub>V</sub> channel activation. These data indicate that K<sub>ATP</sub> channel openers regulate arterial diameter via SUR-dependent and –independent pathways.

Plasma membrane adenosine triphosphate-sensitive potassium (pmK<sub>ATP</sub>) channels couple changes in cellular metabolic activity to membrane electrical excitability (Ashcroft and Ashcroft, 1990). K<sub>ATP</sub> channels are composed of pore-forming K<sub>ir</sub>6.x and regulatory sulfonylurea receptor (SUR) subunits (Aguilar-Bryan et al., 1998). The assembly of four K<sub>ir</sub>6.x and four SUR subunits results in tissue-specific K<sub>ATP</sub> channel complexes with different functional, electrophysiological, and pharmacological properties (Aguilar-Bryan et al., 1998).

SURs are members of the ATP-binding-cassette transporter protein superfamily that are predicted to form 17 transmembrane-spanning helices and 2 intracellular nucleotide-binding domains (Tusnady et al., 1997). Two distinct SUR isoforms (SUR1 and SUR2) have been identified that are ~70 % identical (Aguilar-Bryan et al., 1998). Alternative splicing of the SUR2 gene at the 3' end results in 2 additional isoforms, SUR2A and SUR2B, that have different pharmacological profiles (Isomoto et al., 1996). SURs are the molecular target of pharmacologically diverse and clinically important agonists and antagonists. Sulfonylureas, including glibenclamide and tolbutamide, block K<sub>ATP</sub> channels and are used in the clinic to treat type-2 diabetes because they depolarize pancreatic  $\beta$ -cells and induce insulin secretion (Aguilar-Bryan et al., 1998). K<sub>ATP</sub> channel openers, including pinacidil and cromakalim, activate vascular smooth muscle cell K<sub>ATP</sub> channels, resulting in membrane hyperpolarization and vasodilation (Brayden, 2002). K<sub>ATP</sub> channel openers have been used in the treatment of hypertension and angina, and can mimic ischemic preconditioning, which protects organs, including the heart, against the harmful effects of transient ischemia (Grover, 1994).

Mitochondria K<sub>ATP</sub> (mitoK<sub>ATP</sub>) channels have also been described (O'Rourke, 2004). Several K<sub>ATP</sub> channel openers activate both pmK<sub>ATP</sub> and mitoK<sub>ATP</sub> channels. In cardiac myocytes, diazoxide is a more effective mitoK<sub>ATP</sub> than pmK<sub>ATP</sub> activator, whereas pinacidil similarly activates both pmK<sub>ATP</sub> and mitoK<sub>ATP</sub> channels (Liu et al., 1998). Previously, we have

shown that in rat cerebral artery smooth muscle cells, diazoxide induces a mitochondrial depolarization, leading to reactive oxygen species (ROS) generation (Xi et al., 2005). The mitochondria-derived ROS activate localized intracellular calcium ( $\text{Ca}^{2+}$ ) transients, termed “sparks”, and large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  ( $\text{K}_{\text{Ca}}$ ) channels, leading to vasodilation (Xi et al., 2005). In contrast, pinacidil does not modulate smooth muscle cell mitochondrial potential, ROS, or  $\text{K}_{\text{Ca}}$  channel activity (Xi et al., 2005). This study and earlier investigations which demonstrated that  $\text{K}_{\text{ATP}}$  channel openers activate pm $\text{K}_{\text{ATP}}$  channels indicate that  $\text{K}_{\text{ATP}}$  channel openers can induce vasodilation by activating two different signaling pathways, one of which is mitochondrial and another which involves pm $\text{K}_{\text{ATP}}$  channel activation.

The goal of the present investigation was to study the molecular mechanisms by which  $\text{K}_{\text{ATP}}$  channel openers induce vasodilation. First, we determined whether  $\text{K}_{\text{ATP}}$  channel openers induce vasodilation via a ROS- and  $\text{K}_{\text{Ca}}$  channel-dependent mechanism in systemic (i.e. non-cerebral) arteries and in another species; mouse. Second, we investigated molecular targets for  $\text{K}_{\text{ATP}}$  channel openers in the vasculature. To study this aim, we measured SUR isoforms that are expressed in mesenteric artery smooth muscle cells and used arteries of wild-type ( $\text{SUR2}^{+/+}$ ) and SUR2 deficient ( $\text{SUR2}^{-/-}$ ) mice. We show that mesenteric artery smooth muscle cells of  $\text{SUR2}^{+/+}$  mice express only SUR2B, whereas cells of  $\text{SUR2}^{-/-}$  mice do not express SURs. SUR2B is essential for pinacidil-induced vasodilation, whereas SURs are not required for diazoxide-induced vasodilation. Our data indicate that diazoxide induces vasodilation by inhibiting electron transport chain complex II ( $\text{ETC}_{\text{II}}$ ), leading to ROS-dependent  $\text{K}_{\text{Ca}}$  and voltage-gated potassium ( $\text{K}_{\text{V}}$ ) channel activation. This study identifies two distinct molecular targets by which  $\text{K}_{\text{ATP}}$  channel openers regulate arterial diameter, namely SUR2B and mitochondria  $\text{ETC}_{\text{II}}$ .

## Materials and Methods

**Animals.** Animal protocols used were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution. SUR2<sup>-/-</sup> mice used in the present study were generated by targeted disruption of nucleotide-binding domain (NBD) 1 of SUR2, as previously described (Chutkow et al., 2001). Heterozygous SUR2 deficient mice were then bred onto the FVB mouse substrain for more than 5 generations. Heterozygous mice were interbred or bred into SUR<sup>+/+</sup> mice to generate homozygous null (SUR2<sup>-/-</sup>) or wild type (SUR2<sup>+/+</sup>) mice, respectively. Mice were genotyped using genomic DNA isolated from tail clips of 3-week old littermates.

**Tissue preparation and arterial smooth muscle cell isolation.** Mice (~20 g) were euthanized with an overdose of sodium pentobarbital (i.p., 130 mg kg<sup>-1</sup>). The mesenteric arterial bed was removed from the abdominal cavity, and placed into oxygenated ice-cold (4°C) physiological saline solution (PSS) of the following composition (in mM): 112 NaCl, 4.8 KCl, 24 NaHCO<sub>3</sub>, 1.8 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, and 10 glucose, which was gassed with 21 % O<sub>2</sub>-5 % CO<sub>2</sub>-74 % N<sub>2</sub> to pH 7.4. Third and fourth order mesenteric artery branches (100-150 μm in diameter) were dissected and cleaned of adventitial connective tissue. Smooth muscle cells were enzymatically dissociated from mesenteric arteries using a method similar to that previously described (Jaggar, 2001). Briefly, mesenteric arteries were placed into HEPES-buffered isolation solution (in mM: 55 NaCl, 80 sodium glutamate, 5.6 KCl, 2 MgCl<sub>2</sub>, 10 HEPES, and 10 glucose; pH 7.3 with NaOH) containing 0.3 mg/ml papain, 1 mg/ml dithioerythritol, and 1 mg/ml bovine serum albumin for 20 min (at 37°C) and immediately

transferred to isolation solution containing collagenase F (1 mg/ml), and H (0.5 mg/ml), 100  $\mu$ M CaCl<sub>2</sub> and 1 mg/ml BSA for 8 min (at 37°C). Arteries were subsequently washed in ice-cold isolation solution and triturated using a fire-polished glass pasteur pipette to yield single smooth muscle cells. Smooth muscle cells were maintained in ice-cold (4°C) isolation solution and used for experiments within 2 h after isolation.

**Reverse transcription polymerase chain reaction (RT-PCR).** Arterial smooth muscle cells were placed in a chamber mounted on the stage of an inverted Nikon TS 100 microscope. Spindle-shaped smooth muscle cells were identified and aspirated into a borosilicate glass micropipette as we have done previously (Cheng et al., 2007). ~100 cells were used for each RT-PCR experiment. Total RNA was prepared from arterial smooth muscle cells and whole hearts using Absolutely RNA nanoprep kit (Stratagene, La Jolla, CA) and TRIzol Reagent (Invitrogen, Carlsbad, CA), respectively. RNA concentration and purity was measured using a Beckman DU 650 spectrophotometer (Beckman Coulter, Fullerton, CA). cDNA was synthesized from DNase-treated RNA samples using AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene). Anchored Oligo(dT)<sub>20</sub> Primer (Invitrogen; 100 ng/ $\mu$ l reaction volume) was used for the RT reactions. cDNA products were amplified by nested-PCR using gene-specific oligonucleotide primer pairs. To determine the purity of collected smooth muscle cell populations, cDNA was screened for platelet/endothelial cell adhesion molecule 1 (Pecam-1), myosin, heavy polypeptide 11 (Myh11), ubiquitin carboxy-terminal hydrolase L1 (PGP9.5), and fatty acid binding protein 4 (FABP4), which are endothelial cell, smooth muscle cell, neural cell, and adipocyte markers, respectively. The oligonucleotide primer sequences used for RT-PCR were as follows: SUR1, first-round PCR: 5' - CGAGAGTCCCTTCAATAAGCAA- 3' (forward) and 5' -AATGCTCAAAGAGCTGGCACTC- 3'

(reverse); nested-PCR: 5' -CCCTCTACCAGCACACCAA- 3' (forward) and 5' -  
CAGTCTGCATGAGGCAGGTA- 3' (reverse). SUR2, first -round PCR: 5' -  
TGGACAGAGACAGCTGTTCTG- 3' (forward) and 5' -AGGCAAACACTCCATCTTCCTG- 3'  
(reverse); nested-PCR: 5' -AGCCACTGCTTCCATCGACA- 3' (forward) and 5' -  
CCTCTCTTCATCACAATAACCAG- 3' (reverse).  $\beta$ -actin, first-round PCR: 5' -  
CTACGAGGGCTATGCTCTCC- 3' (forward) and 5' -CTTCTGCATCCTGTCAGCAA- 3'  
(reverse); nested PCR: 5' -GCTACAGCTTACCACCACA- 3' (forward) and 5' -  
AAGGAAGGCTGGAAAAGAGC- 3' (reverse). Pecam-1, first-round PCR: 5' -  
AGAGACGGTCTTGTCGCAGT- 3' (forward) and 5' -AATGGCAATTATCCGCTCTG- 3'  
(reverse); nested PCR: 5' -TGCTCTCGAAGCCCAGTATT- 3' (forward) and 5' -  
TGTGAATGTTGCTGGGTCAT- 3' (reverse). Myh11, first-round PCR: 5' -  
AGCCGGAAAGACAGAGAACA- 3' (forward) and 5' -ACTTTCTGAGCCGCTGTGTT- 3'  
(reverse); nested PCR: 5' -GACAACTCCTCTCGCTTTGG- 3' (forward) and 5' -  
GCTCTCCAAAAGCAGGTCAC- 3' (reverse). PGP9.5, first-round PCR: 5' -  
CGAAGATAGAGCCAAGTGTT- 3' (forward) and 5' -GAAAGTGCTCAGCCTGGTGT- 3'  
(reverse); nested PCR: 5' -GAATGCCCTTTCCAGTGAAC- 3' (forward) and 5' -  
GCTAAAGCTGCAAACCAAGG- 3' (reverse). FABP4, first-round PCR: 5' -  
CAGCCTTTCTCACCTGGAAG- 3' (forward) and 5' -TGGCTCATGCCCTTTCATAA- 3'  
(reverse); nested PCR: 5' -CATCAGCGTAAATGGGGATT- 3' (forward) and 5' -  
TCGACTTTCCATCCCCTTC- 3' (reverse). GeneBank accession numbers used to design  
SUR1, SUR2A, SUR2B,  $\beta$ -actin, Pecam-1, Myh11, PGP9.5, and FABP4 primers were  
NM\_011510, D86037, D86038, NM\_007393, NM\_001032378, NM\_013607, NM\_011670,  
and NM\_024406, respectively. Specific primers for first-round and nested-PCR amplification

of SUR1 were designed to generate cDNA fragments of 413 and 169 bp, respectively. To detect SUR2A and SUR2B transcripts, forward and reverse primers were designed within a region that is conserved in both splice variants. This approach generates different size cDNA fragments for SUR2A (first-round PCR, 451 bp; nested-PCR, 320 bp) and SUR2B (first-round PCR, 275 bp; nested-PCR, 144 bp). PCR amplification was performed in an Eppendorf Mastercycler (Eppendorf, Westbury, NY) with the following reaction conditions: an initial denaturation at 94 °C for 2 min, followed by 40 cycles (denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 45 s), with a final extension at 72 °C for 10 min. Reaction conditions for first-round PCR and nested PCR were the same. PCR products were separated by agarose gel (2%) electrophoresis, followed by ethidium bromide staining, UV transillumination, and documentation on Chemilmager 4000 (Alpha Innotech, San Leandro, CA).

**Pressurized artery diameter measurements.** An arterial segment ~2 mm in length was cannulated at each end in a temperature-controlled perfusion chamber (Living Systems Instrumentation; Burlington, VT). The chamber was continuously perfused with PSS equilibrated with a mixture of 21 % O<sub>2</sub>, 5 % CO<sub>2</sub>, 74 % N<sub>2</sub>, and maintained at 37°C. Arteries were observed with a charge-coupled device camera attached to an inverted microscope (Nikon TE 200). Arterial diameter was measured by using the automatic edge-detection function of IonWizard software (Ionoptix; Milton, MA) and digitized at 1 Hz using a personal computer. Steady-state changes in intravascular pressure were achieved by elevating and lowering an attached reservoir and monitored using a pressure transducer. Intraluminal flow was absent during experiments. Tested compounds were applied via chamber perfusion. Where appropriate, the endothelium was denuded by introducing an air bubble into the artery

lumen for 1 min followed by a wash with PSS. Successful endothelium-denudation was assessed by the lack of arterial dilation to acetylcholine (10  $\mu$ M), an endothelium-dependent vasodilator. Effects of antioxidant, ion channel blockers, and ETC<sub>II</sub> inhibitor on diazoxide- and pinacidil-induced vasodilations were compared with dilations induced by the same K<sub>ATP</sub> channel opener in control in the same artery (i.e. in paired experiments).

**Data Analysis.** Graphpad Instat software (Graphpad Prism, San Diego, CA) was used for statistical analysis. Results are expressed as mean  $\pm$  standard error of the mean. Statistical significance was calculated using Student's t-tests for paired or unpaired data.  $p < 0.05$  was considered significant. The magnitude of myogenic tone was calculated using the following equation: myogenic tone (%) = (1 – active diameter/passive diameter) x 100. Concentration-response curves were fit with a Boltzmann function to derive half-maximal effective concentration (EC<sub>50</sub>) values.

**Chemicals.** Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Atpenin A5 (atpenin), Iberiotoxin, ryanodine, and manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) were purchased from Axxora LLC (San Diego, CA), California Peptide Research, Inc. (Napa, CA), A.G. Scientific, Inc. (San Diego, CA), and Calbiochem (San Diego, CA), respectively.

## Results

### **Diazoxide-induced mesenteric artery vasodilation is endothelium-independent and**

### **occurs due to RyR, K<sub>Ca</sub>, and K<sub>V</sub> channel activation.**

Murine mesenteric arteries were pressurized to 60 mmHg to develop stable myogenic tone. Pinacidil and diazoxide caused vasodilation with EC<sub>50</sub> values of 11 and 53  $\mu$ M, respectively (Fig 1A & B). Endothelium denudation did not alter diazoxide- or pinacidil-induced vasodilation (Fig. 2A). In contrast, MnTMPyP, a superoxide dismutase (SOD) and catalase mimetic, ryanodine, a ryanodine-sensitive Ca<sup>2+</sup> release (RyR) channel blocker, iberiotoxin, a K<sub>Ca</sub> channel blocker, and 4-aminopyridine (4-AP), a K<sub>V</sub> channel blocker, reduced diazoxide-induced vasodilation in the same arteries by ~45-64 % (Fig. 2B). A combination of ryanodine and 4-AP further reduced diazoxide-induced vasodilation by ~77 % . In contrast, iberiotoxin, MnTMPyP, or a combination of ryanodine + 4-AP did not alter pinacidil-induced vasodilation (Fig 2B). Changes in arterial diameter induced by MnTMPyP and channel blockers were (in  $\mu$ m): MnTMPyP, 7  $\pm$  2 (n=19); ryanodine, -10  $\pm$  2 (n=7), iberiotoxin, -7  $\pm$  1 (n=12); 4-AP, -12  $\pm$  4 (n=6); ryanodine + 4-AP, -21  $\pm$  3 (n=19). These data indicate that in murine mesenteric arteries, diazoxide-induced vasodilation occurs due to a ROS elevation, and RyR, K<sub>Ca</sub>, and K<sub>V</sub> channel activation. In contrast, pinacidil-induced vasodilation occurs through a ROS, K<sub>Ca</sub> channel, and K<sub>V</sub> channel-independent mechanism.

### **Wild-type murine mesenteric artery myocytes express only SUR2B, whereas SUR2<sup>-/-</sup>**

**myocytes do not express SUR subunits.** To investigate functional molecular targets for diazoxide and pinacidil in the vasculature, we sought to detect which SUR isoforms are expressed in mesenteric artery smooth muscle cells by using RT-PCR. To determine SUR

subunits that are expressed specifically in mesenteric artery smooth muscle cells, isolated cells were placed in a chamber, visualized using an inverted microscope, and individually collected using a micropipette. Intact mesenteric artery segments contain multiple cell types, as shown by the amplification of transcripts for Pecam-1, Myh11, PGP9.5, and FABP4, which are endothelial-, smooth muscle-, neural-, and adipocyte-specific cell markers. In contrast, isolated mesenteric artery smooth muscle cell cDNA only amplified transcripts for Myh11, but not Pecam-1, PGP9.5, and FABP4. These data indicate that pure smooth muscle cell populations were collected (Fig 3A & B). Although all three sulfonylurea receptor subtypes (SUR1, SUR2A, and SUR2B) were detected in SUR2<sup>+/+</sup> heart lysate cDNA (Fig. 3C), only transcripts for SUR2B were amplified from SUR2<sup>+/+</sup> mouse arterial smooth muscle cell cDNA (Fig. 3D).

**SUR2 is not required for diazoxide-induced vasodilation, but is essential for pinacidil-induced vasodilation.** To determine whether SUR2 expression is required for diazoxide- and pinacidil-induced vasodilation, pressurized (60 mmHg) mesenteric arteries from SUR2<sup>+/+</sup> and SUR2<sup>-/-</sup> mice were studied. Myogenic tone and passive diameter were similar in SUR2<sup>+/+</sup> and SUR2<sup>-/-</sup> arteries (Figs. 4A, B, and C). In SUR2<sup>+/+</sup> arteries, pinacidil (5  $\mu$ M) increased mean diameter by ~ 20  $\mu$ m. In SUR2<sup>-/-</sup> arteries pinacidil-induced dilation was largely absent, although a small dilation ( $2 \pm 1$   $\mu$ m) still occurred (Fig. 5A, C). In contrast, diazoxide (100  $\mu$ M) similarly dilated SUR2<sup>+/+</sup> and SUR2<sup>-/-</sup> arteries (Fig. 5B, D). Collectively, data indicate that SUR2 is required for pinacidil-induced vasodilation and that diazoxide-induced vasodilation does not result from the activation of SUR-containing K<sub>ATP</sub> channels.

**Electron transport chain complex II inhibition produces vasodilation mechanistically**

**similar to diazoxide and attenuates diazoxide-induced vasodilation.** We tested the

hypothesis that diazoxide induces vasodilation by inhibiting ETC<sub>II</sub> (Hanley et al., 2002).

Atpenin (1  $\mu$ M), a potent and selective ETC<sub>II</sub> inhibitor (Miyadera et al., 2003), increased the mean diameter of pressurized (60 mmHg) mesenteric arteries by  $\sim$ 10  $\mu$ m, which is similar to

the dilation induced by diazoxide (Fig. 5D, 6A, and B). MnTMPyP or a combination of

ryanodine + 4-AP reduced mean atpenin-induced vasodilation by  $\sim$ 52 and 60 %, respectively

(Fig. 6C). Atpenin also reduced mean diazoxide-induced vasodilation by  $\sim$  73 %, but did not

alter vasodilation induced by pinacidil (Fig. 7A, B). These data indicate that atpenin and

diazoxide regulate arterial diameter through similar mechanisms, implicate the arterial

smooth muscle cell ETC<sub>II</sub> as the molecular target through which diazoxide induces

vasodilation, and indicate that pinacidil does not induce vasodilation by inhibiting the ETC.

## Discussion

In the present study, we investigated functional molecular targets for  $K_{ATP}$  channel openers in smooth muscle cells of small, resistance-size, mesenteric arteries. We demonstrate that diazoxide-induced dilation of pressurized mesenteric arteries is independent of SURs but involves smooth muscle cell ETC inhibition, a ROS elevation and RyR,  $K_{Ca}$ , and  $K_V$  channel activation. In contrast, pinacidil-induced vasodilation requires SUR2B and does not result from ROS generation, or RyR,  $K_{Ca}$  or  $K_V$  channel activation. These findings indicate that pinacidil and diazoxide induce vasodilation by two distinct mechanisms; one that requires SUR2B and another that targets the mitochondria ETC.

$K_{ATP}$  channels are expressed in the vascular endothelium and may modulate vasodilation in response to shear stress, acidosis, and vasodilators, including adenosine (Yoshida et al., 2004; Brayden, 2002). Here, pinacidil and diazoxide similarly dilated endothelium-intact and -denuded arteries. These data indicate that in mesenteric arteries, endothelial cell-mediated mechanisms do not significantly contribute to the vasodilation induced by these  $K_{ATP}$  channel openers.

In arterial smooth muscle cells,  $Ca^{2+}$  sparks occur due to the opening of several RyR channels on the sarcoplasmic reticulum. A  $Ca^{2+}$  spark activates several  $K_{Ca}$  channels, resulting in a transient  $K_{Ca}$  current. Asynchronous transient  $K_{Ca}$  currents within the arterial wall induce membrane hyperpolarization, which reduces voltage-dependent  $Ca^{2+}$  channel activity, leading to vasodilation (Jaggar et al., 2000). Previously, we showed that in smooth muscle cells of rat resistance-size cerebral arteries, diazoxide induces mitochondrial depolarization, leading to a ROS elevation that activates  $Ca^{2+}$  sparks and  $K_{Ca}$  channels (Xi et al., 2005). The resulting membrane hyperpolarization leads to vasodilation. In contrast, in rat cerebral arteries, pinacidil-induced vasodilation does not result from mitochondrial ROS

generation or  $\text{Ca}^{2+}$  spark and  $\text{K}_{\text{Ca}}$  channel activation (Xi et al., 2005). Collectively, data here and in our previous study indicate that in both rat cerebral and mouse mesenteric arteries, diazoxide-induced vasodilation occurs due to mitochondria-derived ROS-mediated RyR and  $\text{K}_{\text{Ca}}$  channel activation, whereas pinacidil-induced vasodilation occurs via a ROS,  $\text{Ca}^{2+}$  spark, and  $\text{K}_{\text{Ca}}$  channel-independent mechanism (Xi et al., 2005). These studies suggest that the mechanisms by which diazoxide and pinacidil induce vasodilation are similar in arteries from different species and vascular beds.

Ryanodine and iberiotoxin reduced diazoxide-induced vasodilation less in mesenteric arteries than in cerebral arteries (Xi et al., 2005). These data suggested that in addition to  $\text{K}_{\text{Ca}}$  channel activation, other mechanisms mediated diazoxide-induced vasodilation in mesenteric arteries. In rat mesenteric and canine coronary artery smooth muscle cells, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) activated 4-AP-sensitive  $\text{K}^+$  currents (Gao et al., 2003; Rogers et al., 2007). Similarly, 4-AP blocked  $\text{H}_2\text{O}_2$ -induced relaxation of mesenteric and coronary artery rings (Gao et al., 2003; Rogers et al., 2006). These studies suggested that ROS relaxed mesenteric and coronary arteries by activating  $\text{K}_{\text{V}}$  channels. Therefore, we determined whether diazoxide-induced ROS dilated mesenteric arteries by activating 4-AP-sensitive  $\text{K}_{\text{V}}$  channels. 4-AP significantly reduced diazoxide-induced vasodilation in murine mesenteric arteries, and a combination of 4-AP and ryanodine, which would simultaneously block  $\text{K}_{\text{V}}$  and RyR channels, reduced diazoxide-induced vasodilation more than did each blocker when applied alone. These data suggest that in mesenteric arteries, diazoxide-induced ROS generation activates both  $\text{K}_{\text{Ca}}$  and  $\text{K}_{\text{V}}$  channels, resulting in membrane hyperpolarization and vasodilation. In contrast, iberiotoxin and a combination of ryanodine and 4-AP did not alter pinacidil-induced vasodilation, indicating that this response is RyR,  $\text{K}_{\text{Ca}}$ , and  $\text{K}_{\text{V}}$  channel-independent. In cerebral artery myocytes, diazoxide (100  $\mu\text{M}$ )-induced

$K_{Ca}$  channel activation was abolished by CCCP, a mitochondrial uncoupler (Xi et al., 2005). Thus, diazoxide activates smooth muscle cell  $K_{Ca}$  channels by a mitochondria-dependent mechanism (Xi et al., 2005). We did not determine here whether diazoxide directly activates  $K_V$  channels, but previous studies have demonstrated that  $K_V$  channels are activated by ROS, supporting our proposal (Gao et al., 2003;Rogers et al., 2006;Rogers et al., 2007).

ROS have been demonstrated to induce both vasoconstriction and vasodilation (Lyle and Griendling, 2006). These apparently disparate findings may relate to the vascular tissue and species studied, the experimental techniques used to measure contractility, whether ROS were applied exogenously or generated endogenously, and whether the ROS elevation was acute or prolonged. In general, our data are consistent with previous evidence that  $K_V$  channels mediate ROS-induced relaxation in non-pressurized coronary and mesenteric artery rings, although differences are also apparent. In non-pressurized mesenteric and coronary artery rings that were pre-contracted with U46619 and phenylephrine,  $K_{Ca}$  channel activation did not mediate  $H_2O_2$ -induced relaxation (Gao et al., 2003;Rogers et al., 2006). Intravascular pressure activates  $Ca^{2+}$  sparks, whereas vasoconstrictors block  $Ca^{2+}$  sparks and thus,  $K_{Ca}$  channels (Jaggar, 2001;Jaggar and Nelson, 2000). The inability of  $K_{Ca}$  channel blockers to prevent ROS-induced relaxation in mesenteric and coronary artery rings (Gao et al., 2003;Rogers et al., 2006) likely reflects a combination of the absence of pressure-induced  $K_{Ca}$  channel activation and  $K_{Ca}$  channel inhibition by the vasoconstrictor used to induce tone. Conceivably, mitochondria-derived ROS and exogenous  $H_2O_2$  application may also activate different  $K^+$  channel subtypes. Regardless of these differences, our data indicate that  $K_V$  channel activation makes a larger contribution to diazoxide-induced, ROS-mediated vasodilation in pressurized murine mesenteric arteries than in pressurized rat cerebral

arteries. Whether this mechanistic difference is due to the species or anatomical origin of the arteries remains to be determined.

Previous studies have identified SUR isoforms expressed in intact vessels (Cao et al., 2002; Ploug et al., 2006). Since SURs are expressed in multiple vascular wall cell types, including endothelial cells and perivascular neurons, it was unclear which SUR isoforms were present specifically in smooth muscle cells (Yoshida et al., 2004; Aguilar-Bryan et al., 1998). We performed RT-PCR on isolated smooth muscle cells to avoid mRNA contamination from other vascular wall cell types. As a positive control, SUR subtypes expressed in SUR<sup>+/+</sup> whole heart were examined. Transcripts for SUR1, SUR2A, and SUR2B were amplified from whole heart cDNA, consistent with previous studies (Isomoto et al., 1996; Morrissey et al., 2005), whereas only SUR2B was detected in mouse arterial smooth muscle cells. These data indicate that SUR2B is the only SUR subtype that is expressed in mesenteric artery smooth muscle cells.

Plasma membrane K<sub>ATP</sub> currents have been recorded in a wide variety of vascular smooth muscle cell types, including cells from mesenteric artery (Quayle et al., 1995; Brayden, 2002). We have previously shown that genetic ablation of SUR2 abolishes vascular smooth muscle cell K<sub>ATP</sub> currents (Kakkar et al., 2006). In the present study, myogenic tone and passive diameter were similar in SUR2<sup>-/-</sup> and SUR2<sup>+/+</sup> arteries. These data suggest that K<sub>ATP</sub> channels do not contribute significantly to resting myogenic tone and that genetic ablation of SUR2 does not result in significant remodeling of the mesenteric vasculature. pmK<sub>ATP</sub> channels formed from K<sub>i,r</sub>6.2/SUR1 or K<sub>i,r</sub>6.2/SUR2B subunits were activated by diazoxide and pinacidil (Babenko et al., 1998). In contrast, K<sub>i,r</sub>6.2/SUR2A channels were activated by pinacidil and insensitive to diazoxide (Babenko et al., 1998). These data suggest that pinacidil activates K<sub>ATP</sub> channels containing all SUR subtypes

whereas diazoxide activates only channels containing SUR1 or SUR2B. In the present study, pinacidil-induced dilation of pressurized SUR2<sup>-/-</sup> mesenteric arteries was virtually absent, indicating that SUR2B is the primary vasodilatory target for pinacidil. However, a small pinacidil-induced dilation was present in SUR2<sup>-/-</sup> arteries, indicating that a SUR-independent target also exists. Since pinacidil-induced vasodilation was not attenuated by iberiotoxin, MnTMPyP, or ryanodine plus 4-AP, the signaling pathway mediating the SUR-independent vasodilation to pinacidil is unclear. In contrast, diazoxide similarly dilated SUR2<sup>+/+</sup> and SUR2<sup>-/-</sup> arteries. Collectively, these findings indicate that plasma membrane SUR2B-containing K<sub>ATP</sub> channels mediate pinacidil-induced vasodilation. In contrast, diazoxide-induced vasodilation is entirely independent of SUR-containing plasma membrane K<sub>ATP</sub> channels.

MitoK<sub>ATP</sub> channels have been studied in several tissues, including liver, heart, and brain (O'Rourke, 2004). Although heterologous expression of K<sub>ir</sub>6.1 and SUR1 subunits generates currents with similar pharmacology to mitoK<sub>ATP</sub>, the precise molecular composition of mitoK<sub>ATP</sub> is unclear (Liu et al., 2001). Western blot analysis of mitochondria isolated from liver and skeletal muscle detected K<sub>ir</sub>6.1 (Suzuki et al., 1997). A low affinity binding site for glibenclamide in purified mitochondria preparations has been proposed to be a mitochondrial SUR (Szewczyk et al., 1997). Furthermore, mitoK<sub>ATP</sub> reconstituted in liposomes consists of 55- and 63-kDa proteins that may be mitoK<sub>ir</sub> and mitoSUR, respectively (Mironova et al., 2004; Bajgar et al., 2001). These studies suggest that mitoK<sub>ATP</sub> channels may be similar to pmK<sub>ATP</sub> channels and consist of K<sub>ir</sub> and SUR subunits. Other studies have suggested that mitochondrial respiratory chain complexes are targets for diazoxide-induced cardioprotection (Ovide-Bordeaux et al., 2000; Droese et al., 2006). Mitochondrial ETC<sub>II</sub> (succinate dehydrogenase) and four mitochondrial inner membrane proteins (mitochondrial ATP-binding cassette protein 1 (mABC1), phosphate carrier, adenine nucleotide translocator, and ATP

synthase) reconstituted cardiac myocyte mitoK<sub>ATP</sub> channels that were activated by diazoxide and ETC<sub>II</sub> inhibitors and blocked by glibenclamide and 5-HD (Ardehali et al., 2004). Thus, the ETC<sub>II</sub> may be a functional component of mitoK<sub>ATP</sub> channels that contain mABC1, but not SURs (Ardehali et al., 2004). K<sub>ATP</sub> channel-independent modulation of mitochondrial metabolism by glibenclamide has also been described (Engbersen et al., 2005), and 5-HD is a substrate for acyl-coA synthetase (Hanley et al., 2002). We have previously reported that glibenclamide, a pmK<sub>ATP</sub> and mitoK<sub>ATP</sub> channel blocker and 5-HD, a mitoK<sub>ATP</sub> channel blocker, attenuated diazoxide-induced mitochondrial depolarization and transient K<sub>Ca</sub> current activation in cerebral artery myocytes (Xi et al., 2005). Collectively, data here and in our previous study indicate that SUR subunits do not mediate diazoxide-induced mitochondrial depolarization, ROS generation, transient K<sub>Ca</sub> current activation, and vasodilation, or inhibition of these effects by glibenclamide and 5-HD (Xi et al., 2005). In summary, our data provide evidence that if mitoK<sub>ATP</sub> channels in arterial myocytes are targets of diazoxide and K<sub>ATP</sub> channel blockers, these channels do not contain SUR subunits.

The ETC is a primary source of ROS in many cell types, including smooth muscle cells (Lesnefsky et al., 2001). Both diazoxide and pinacidil stimulate ROS generation in cardiac myocytes, which may underlie their cardioprotective effects (Forbes et al., 2001; Lesnefsky et al., 2001; Lember et al., 2003). In contrast, diazoxide elevated mitochondria-derived ROS in rat cerebral artery smooth muscle cells, whereas pinacidil had no effect (Xi et al., 2005). To investigate if diazoxide dilated mesenteric arteries by inhibiting ETC<sub>II</sub>, we studied diameter regulation by atpenin. Atpenin blocks ETC<sub>II</sub> at concentrations ~3-orders of magnitude lower than other commonly used ETC<sub>II</sub> inhibitors, including carboxin and TFA (Miyadera et al., 2003). In the present study, 1 μM atpenin dilated pressurized mesenteric arteries similarly to 100 μM diazoxide. We also tested the hypothesis that similarly to diazoxide, ETC<sub>II</sub> inhibition

by atpenin causes vasodilation via ROS and RyR and  $K_V$  channel activation. MnTMPyP or a combination of ryanodine and 4-AP caused a similar reduction in atpenin- and diazoxide-induced induced vasodilation. Furthermore, atpenin attenuated diazoxide-induced vasodilation, but did not alter pinacidil-induced vasodilation. These data suggest that ETC<sub>II</sub> inhibition by either atpenin or diazoxide dilates mesenteric arteries via a ROS,  $Ca^{2+}$  spark,  $K_{Ca}$ , and  $K_V$  channel pathway. Our results also indicate that the mitochondrial ETC and not a SUR-containing  $K_{ATP}$  channel, is the functional molecular target for diazoxide-induced vasodilation. Since diazoxide targets the ETC, ATP/ADP may decrease. This would be expected to activate  $K_{ATP}$  channels and contribute to the dilation. However, diazoxide similarly dilated SUR2<sup>+/+</sup> and SUR2<sup>-/-</sup> arteries. Therefore, any ATP/ADP shift does not appear to be sufficient to activate  $K_{ATP}$  channels. These data indicate that pinacidil and diazoxide induce vasodilation by SUR-dependent and independent pathways, respectively. Other structurally unrelated KCOs, including minoxidil sulfate, nicorandil, and aprikalim, may also act through these pathways and induce vasodilation by other mechanisms including nitric oxide generation (Rosenblum, 2003).

In conclusion, our study indicates that pinacidil and diazoxide dilate resistance-size mesenteric arteries by two molecularly distinct signaling pathways. Pinacidil-induced vasodilation requires SUR2B. In contrast, diazoxide-induced vasodilation occurs independently of SUR-containing  $K_{ATP}$  channels and involves mitochondrial ETC<sub>II</sub> inhibition, a smooth muscle cell ROS elevation and RyR,  $K_{Ca}$  channel, and  $K_V$  channel activation.

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## **Footnotes**

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## Figure Legends

**Figure 1. Concentration-dependent dilation by pinacidil and diazoxide in pressurized (60 mmHg) murine mesenteric arteries.** Concentration-response effect of **A**, pinacidil  $EC_{50} = 11 \mu\text{M}$  ( $n=8$ ), and **B**, diazoxide  $EC_{50} = 53 \mu\text{M}$  ( $n=6$ ).

**Figure 2. Diazoxide-induced mesenteric artery dilation is endothelium-independent and attenuated by RyR,  $K_{Ca}$ , and  $K_V$  channel blockers.** **A**, Diameter responses to acetylcholine (ACh,  $10 \mu\text{M}$ ;  $n=7$ ), diazoxide (Diaz,  $100 \mu\text{M}$ ; endothelium-intact:  $n=28$ , endothelium-denuded:  $n=7$ ) and pinacidil (Pinac,  $5 \mu\text{M}$ ; endothelium-intact:  $n=19$ , endothelium-denuded:  $n=7$ ) in pressurized (60 mmHg) mesenteric arteries. **B**, Diameter responses to diazoxide and pinacidil in endothelium-intact arteries. Filled bars illustrate the average effect of MnTMPyP ( $100 \mu\text{M}$ ,  $n=6$ ), ryanodine ( $10 \mu\text{M}$ ,  $n=6$ ), iberiotoxin ( $100 \text{ nM}$ ,  $n=6$ ), 4-AP ( $1 \text{ mM}$ ,  $n=6$ ), and ryanodine + 4-AP ( $n=7$ ) on dilation induced by diazoxide ( $100 \mu\text{M}$ ). Empty bars show the mean effects of iberiotoxin ( $100 \text{ nM}$ ,  $n=6$ ), MnTMPyP ( $100 \mu\text{M}$ ,  $n=7$ ) and ryanodine + 4-AP ( $n=7$ ) on dilation induced by pinacidil ( $5 \mu\text{M}$ ). \*\*  $p < 0.05$  when compared with endothelium-intact arteries; \*  $p < 0.05$  when compared with the control.

**Figure 3.  $SUR2^{+/+}$  murine mesenteric artery myocytes express SUR2B, whereas  $SUR2^{-/-}$  myocytes do not express SUR subunits.** **A**, Mesenteric artery segments express transcripts for Pecam-1, Myh11, PGP9.5, and FABP4. **B**, In contrast, isolated, selected mesenteric artery smooth muscle cells only express transcript for Myh11. **C**, RT-PCR indicates that transcripts for SUR1, SUR2A, and SUR2B are amplified from cDNA generated from wild-type heart lysate (control;  $n=4$  for each). **D**, mesenteric artery smooth muscle cells

isolated from SUR2<sup>+/+</sup> mice express only SUR2B, whereas mesenteric artery smooth muscle cells from SUR2<sup>-/-</sup> mice do not express any SURs (n=4 for each). Transcript-specific primers for Pecam-1, Myh11, PGP9.5, FABP4, SUR1, SUR2A, SUR2B, and  $\beta$ -actin generated cDNA fragments of 176, 201, 219, 182, 169, 320, 144, and 208 bp, respectively.

**Figure 4. Myogenic response and passive diameter are similar in mesenteric arteries**

**of SUR2<sup>+/+</sup> and SUR2<sup>-/-</sup> mice.** **A**, Representative traces illustrating that an elevation in intravascular pressure from 10 to 60 mmHg induces a similar myogenic constriction in SUR2<sup>+/+</sup> and SUR2<sup>-/-</sup> arteries. **B**, Mean myogenic tone in SUR2<sup>+/+</sup> (n=49) and SUR2<sup>-/-</sup> arteries (n=24) pressurized to 60 mmHg. **C**, Mean passive diameter of SUR2<sup>+/+</sup> (n=49) and SUR2<sup>-/-</sup> arteries (n=24), as determined in a Ca<sup>2+</sup>-free bath solution.

**Figure 5. SUR deficiency inhibits pinacidil-induced dilation, but does not alter**

**diazoxide-induced dilation in mesenteric arteries.** **A** and **B**, Representative traces illustrating a pinacidil (5  $\mu$ M) and diazoxide (100  $\mu$ M)-induced dilation in SUR2<sup>+/+</sup> and SUR2<sup>-/-</sup> arteries. **C** and **D**, Mean dilation induced by pinacidil (SUR2<sup>+/+</sup>, n=19; SUR2<sup>-/-</sup>, n=16) and diazoxide (SUR2<sup>+/+</sup>, n=28; SUR2<sup>-/-</sup>, n=15). \**p* < 0.05 when compared with SUR2<sup>+/+</sup>.

**Figure 6. Atpenin, an ETC<sub>II</sub> inhibitor, dilates pressurized arteries and this effect is**

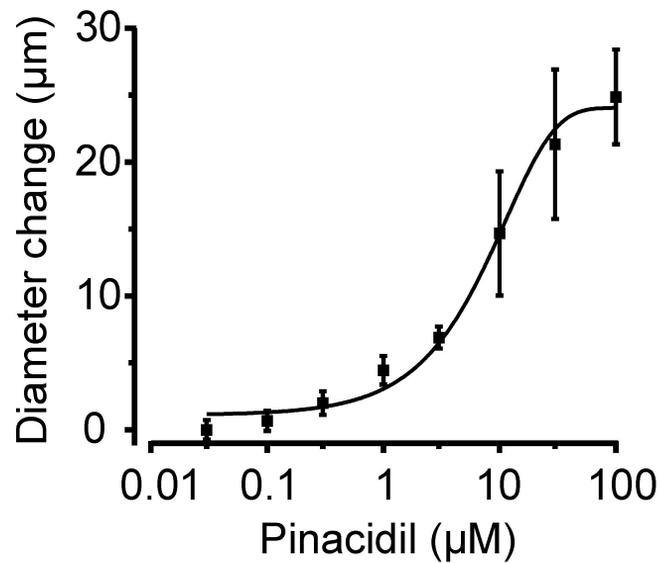
**attenuated by MnTMPyP and ryanodine + 4-AP.** **A**, Representative trace illustrating atpenin (1  $\mu$ M)-induced vasodilation and dilation induced by removal of bath Ca<sup>2+</sup> in the same artery. **B**, Mean arterial dilation to atpenin (1  $\mu$ M, n=10). **C**, MnTMPyP (100  $\mu$ M, n=6) and ryanodine + 4-AP (n=5) attenuate atpenin (1  $\mu$ M)-induced dilation. \**p* < 0.05 when compared with the control.

**Figure 7. Atpenin reduces diazoxide-, but not pinacidil-induced, vasodilation. A,**

Representative traces illustrating vasodilation to diazoxide (100  $\mu$ M) and pinacidil (5  $\mu$ M) in the same arteries in control (black trace) and in the presence of atpenin (1  $\mu$ M, gray trace).

**B,** Mean effects of atpenin on dilation induced by diazoxide (100  $\mu$ M; n=9) and pinacidil (5  $\mu$ M; n=7). \* $p < 0.05$  when compared with the control.

A



B

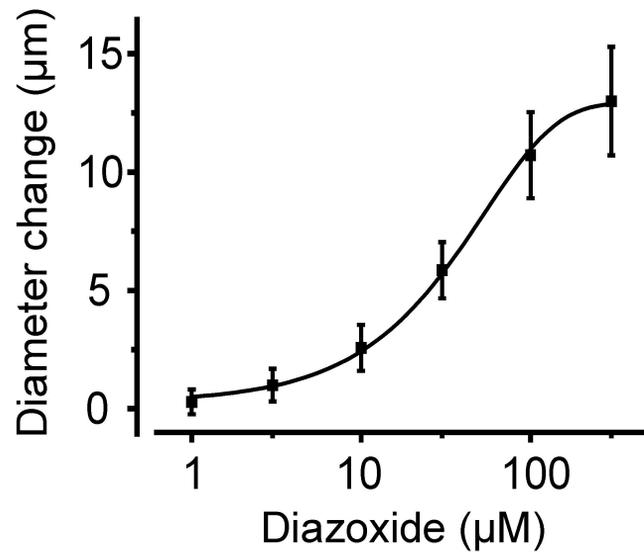


Figure 1

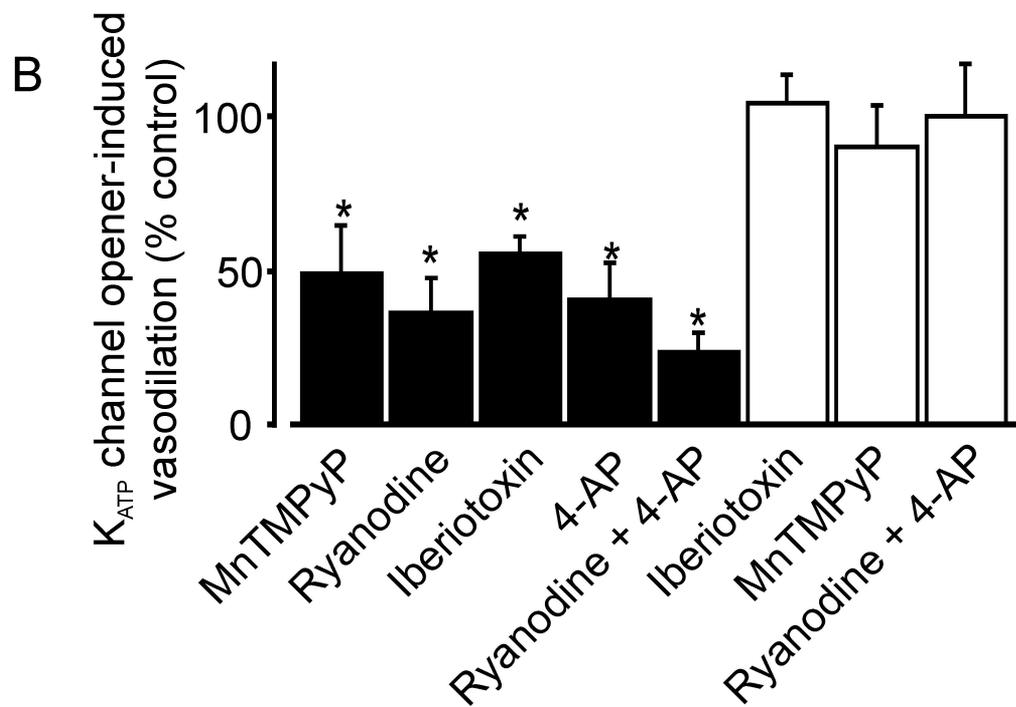
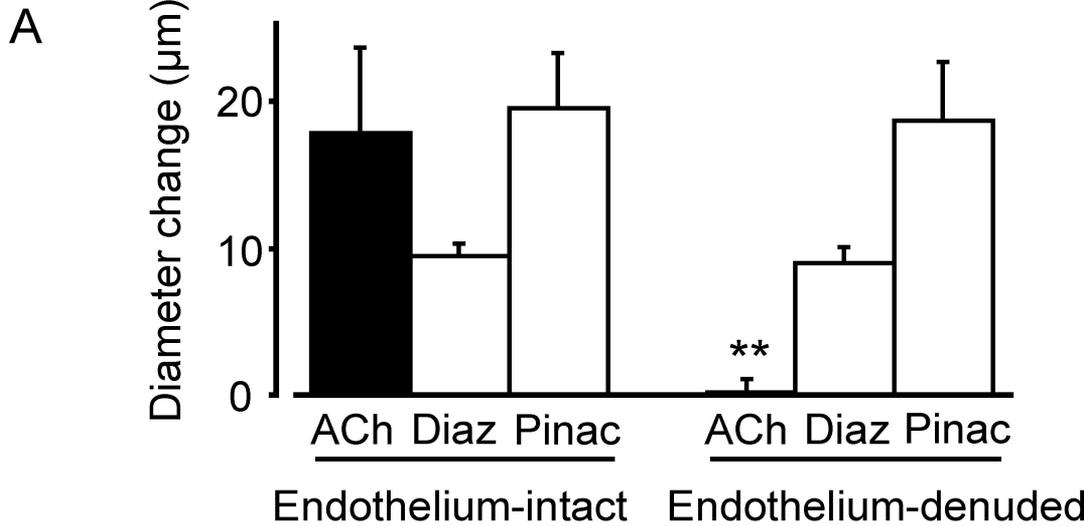


Figure 2

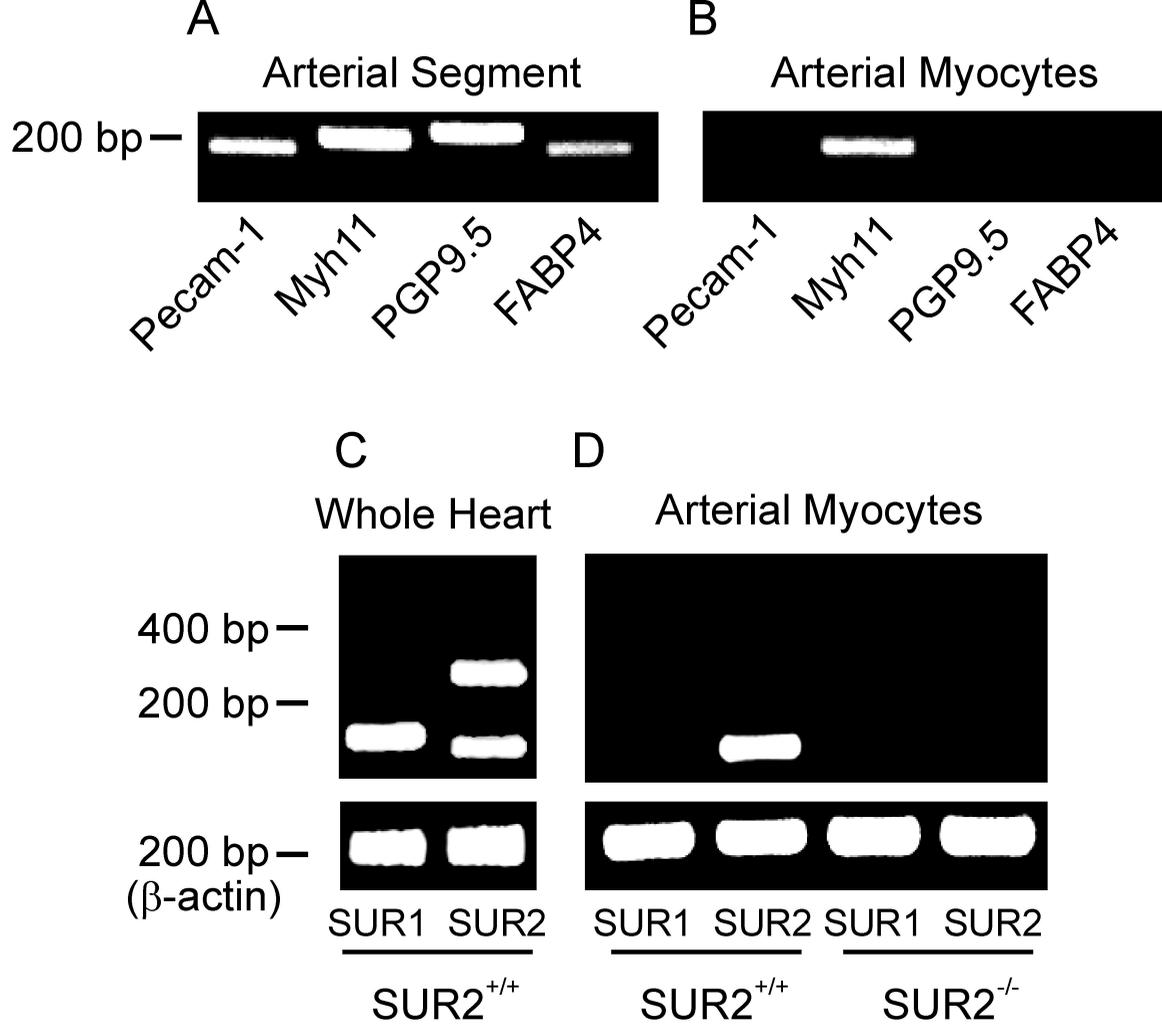


Figure 3

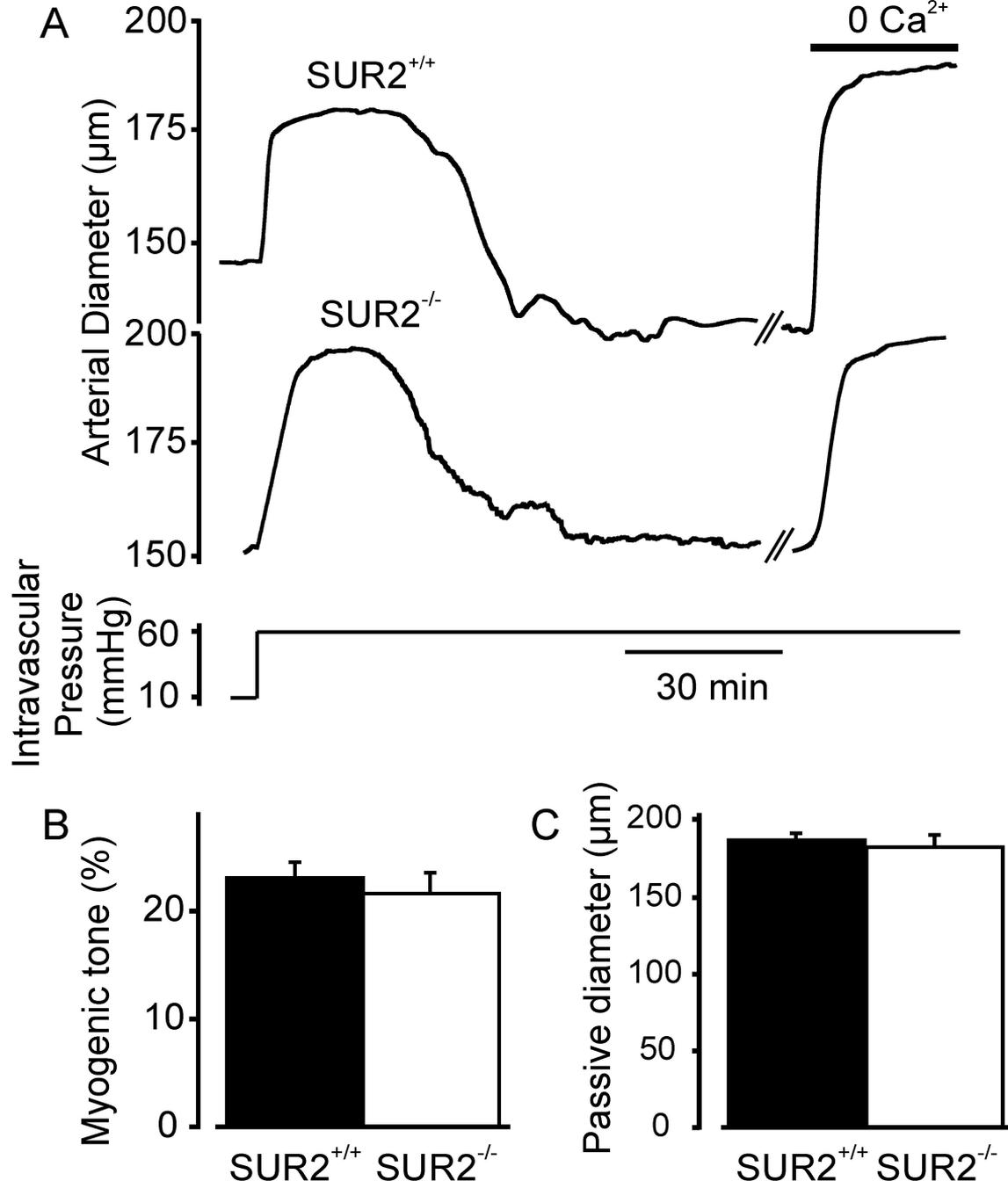


Figure 4

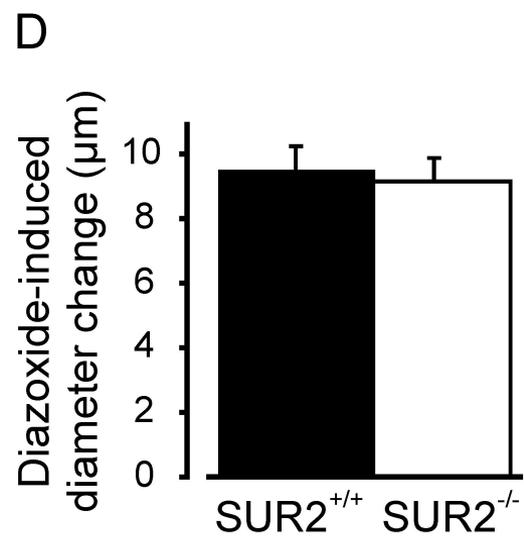
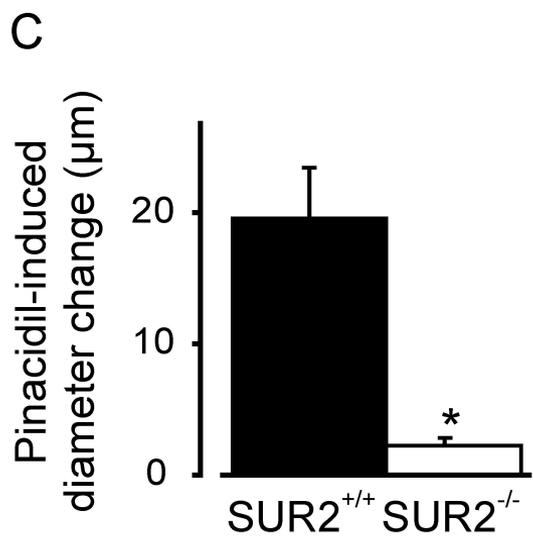
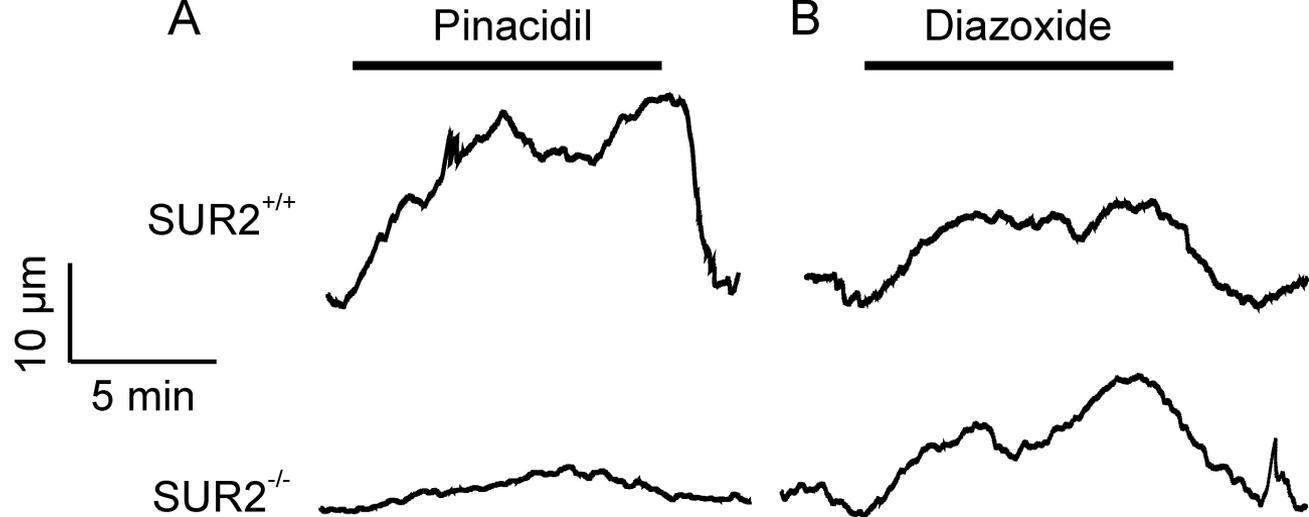


Figure 5

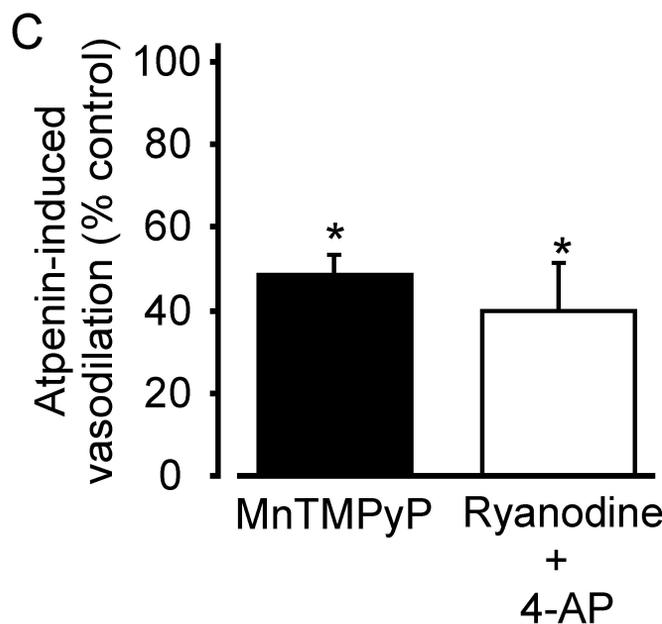
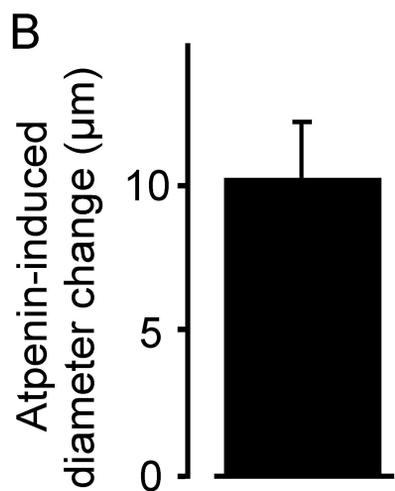
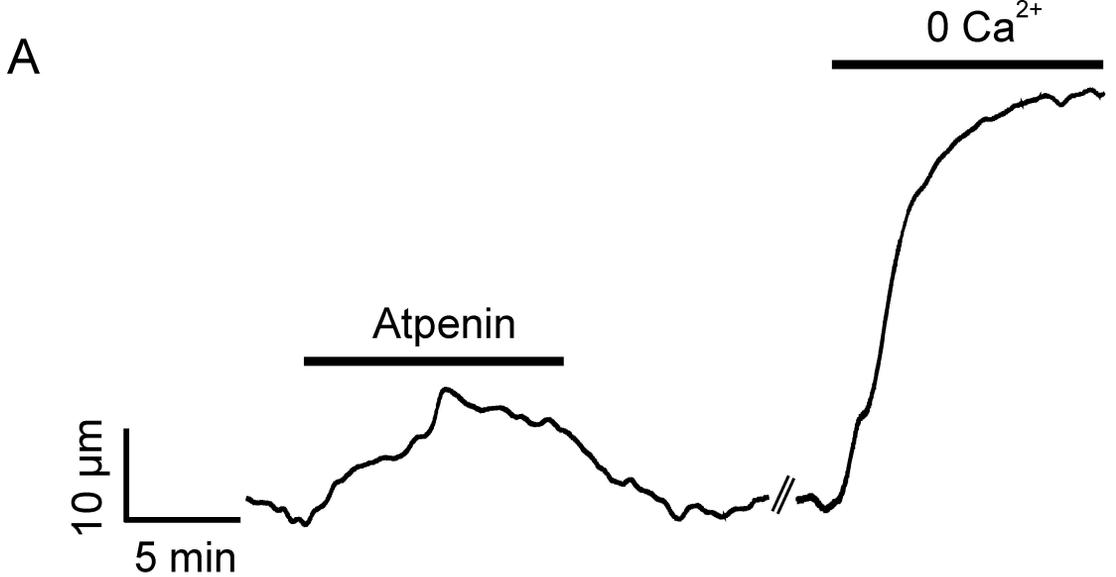


Figure 6

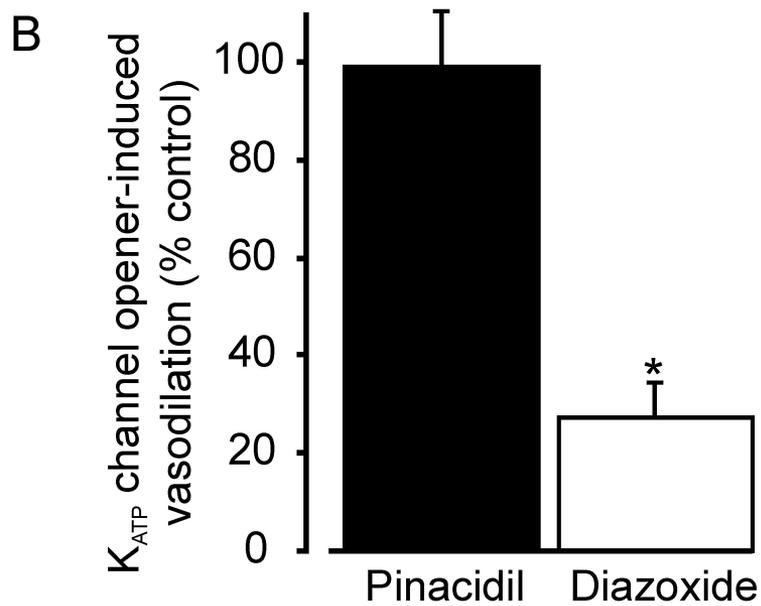
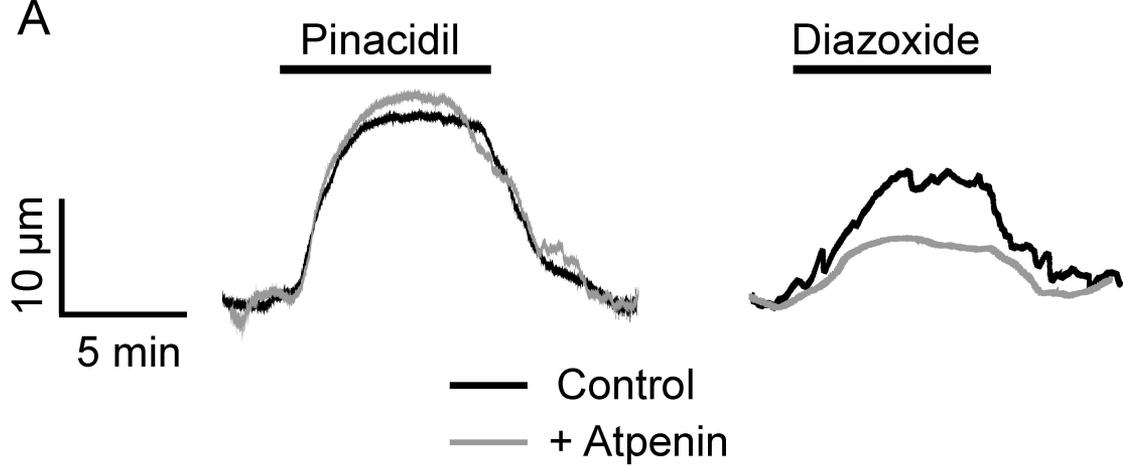


Figure 7