Contribution of Kv7 Channels to Basal Coronary Flow and Active Response to Ischemia

Saereh Khanamiri, Ewa Soltysinska, Thomas A. Jepps, Bo H. Bentzen, Preet S. Chadha, Nicole Schmitt, Iain A. Greenwood, Søren-Peter Olesen

ABSTRACT—The goal of the present study was to determine the role of KCNQ-encoded Kv7 channels (Kv7 channels) in the passive and active regulation of coronary flow in normotensive and hypertensive rats. In left anterior descending coronary arteries from normotensive rats, structurally different Kv7.2 to 7.5 activators produced relaxations, which were considerably less in arteries from hypertensive rats and were not mimicked by the Kv7.1-specific activator R-L3. In isolated, perfused heart preparations, coronary flow rate increased in response to the Kv7.2 to 7.5 activator (S)-1 and was diminished in the presence of a Kv7 inhibitor. The expression levels of KCNQ1–5 and their known accessory KCNE1–5 subunits in coronary arteries were similar in normotensive and hypertensive rats as measured by quantitative polymerase chain reaction. However, Kv7.4 protein expression was reduced in hypertensive rats. Application of adenosine or A2A receptor agonist CGS-21680 produced concentration-dependent relaxations of coronary arteries from normotensive rats, which were attenuated by application of Kv7 inhibitors. Kv7 blockers also attenuated the ischemia-induced increase in coronary perfusion in Langendorff studies. Overall, these data establish Kv7 channels as crucial regulators of coronary flow at resting and after hypoxic insult. (Hypertension. 2013;62:1090-1097.) • Online Data Supplement

KEY WORDS: adenosine • coronary vessels • hyperemia • hypertension • muscle, smooth, vascular • vasodilation

Failure of the coronary circulation to meet the constant demands of the cardiomyocytes results in ischemic heart disease or infarction. Determining the factors that regulate coronary blood flow is, therefore, crucial for understanding pathophysiological manifestations and for the development of new therapeutic strategies. Voltage-gated potassium channels (Kv) have been implicated in the control of the coronary circulation and reactive hyperemia,1,2 but little is known about the specific molecular components. Kv channels encoded by KCNQ1–5 (Kv7.1–Kv7.5) are important regulators of the smooth muscle resting membrane potential and contractility in several different rodent and human arteries,3–10 which are known to be compromised in animal models of primary and secondary hypertension.3,6 These channels, in particular Kv7.4, are also functional end points in β-adrenoceptor-mediated relaxations.3,11 These combined observations provide a mechanism by which arteries become relatively resistant to endogenous vasorelaxants. However, little is known about Kv7 expression levels or the functional importance of these channels in coronary arteries. Consequently, we determined the expression profiles of KCNQ genes and the functional impact of Kv7 channel modulators in left anterior descending (LAD) coronary arteries. We also ascertained whether Kv7 channels contribute to relaxations produced by adenosine, which is released rapidly after myocardial ischemia in vivo and acts as a local metabolic regulator of coronary flow.12,13 This study demonstrates that Kv7 channels are key regulators of coronary flow at rest, and they also contribute to adenosine-mediated dilatations and the active response to ischemia.

METHODS

For more complete descriptions, see the online-only Data Supplement.

ANIMALS

The study complies with the European Community Guidelines for the Care and Use of Experimental Animals and was approved by the Animal Ethics Screening Committee in Denmark (license number: 2010/561–1799) and by the UK Animal (Scientific Procedures) Act 1986. Male, normotensive Wistar Hannover rats and spontaneously hypertensive rats (SHRs), aged 11 to 16 weeks, were purchased from...
Functional Studies
Isometric tension studies were performed on isolated first-, second-, and third-order LAD coronary artery branches (2–4 vessels per animal). Measurements of vessel diameter and basal tone after normalization are illustrated in Figure S1 in the online-only Data Supplement. Coronary flow was measured in isolated perfused heart preparations. Cardiac ischemia was induced by stopping perfusion for 90 s.

Expression Studies
Expression profiles of KCNQ1–5 and the auxiliary subunits KCNE1–5 were measured using quantitative polymerase chain reaction (PCR) in LAD coronary arteries from both normotensive rats and SHRs. Primer sequences are listed in Table S1. A S1

Protein expression of K\textsubscript{7} 7.4 in coronary arteries from the first-, second-, and third-order LAD branches of normotensive and hypertensive rats was determined by Western blotting.

Statistical Analysis
A 2-way ANOVA test followed by a Bonferroni post hoc test was used for comparing the effects of K\textsubscript{7} 7 activators and blockers in normotensive and hypertensive animals. Effects of adenosine, A2 (CGS-21680), cAMP analogue (8-Br-cAMP), and protein kinase A inhibitors in normotensive rats were compared with the dimethyl sulfoxide (DMSO) control using a 2-way ANOVA test followed by a Bonferroni post hoc test. A 1-way ANOVA test followed by Tukey’s multiple comparison post-test was used to compare the effect of DMSO and K\textsubscript{7} 7 blockers on resting coronary flow. An unpaired t test was used to compare the effect of (S)-1 and (S)-1+K\textsubscript{7} 7 channel inhibitor on isolated hearts and to analyze the effect of K\textsubscript{7} 7 blockers on reactive hyperemia indices. In all tests, P values <0.05 were considered to be significant.

Drugs
(S)-1, BMS-254352, and retigabine were synthesized at NeuroSearch A/S (Ballerup, Denmark). U46619, linopirdine, XE-991, isoproterenol, adenosine, CGS-21680, 8-Br-cAMP, KT5720, H89, and SNP were purchased from Sigma-Aldrich (Brøndby, Denmark). R-L3 was purchased from Tocris (Abingdon, United Kingdom) and HMR1556 was from Janssen Pharmaceuticals (Belgium).

Results
Cumulative application of the pan-K\textsubscript{7}7 channel inhibitors XE-991 or linopirdine (1, 3, 5, or 10 μmol/L) to LAD coronary arteries isolated from normotensive rats produced a concentration-dependent contraction that was not observed in coronary arteries from SHRs (Figure 1) and not seen with the K\textsubscript{7}7.1-specific blocker HMR1556 (Figure S2). To calibrate the system, we used cumulative addition of U46619 within a concentration range of 0.1 to 10 nmol/L in normotensive rat and SHR vasculature to match the same contraction achieved by applying 60 μmol/L KCl (Figure S3). Coronary arteries precontracted by 1 nmol/L U46619 were relaxed by structurally different K\textsubscript{7}7.2 to 7.5 channel activators (S)-1, BMS-254352, and retigabine with an order of potency (S)-1=BMS-204352>retigabine (Figure 2B). All relaxations to these agents were abrogated by preincubation with 10 μmol/L linopirdine (Figure 2D) but were not affected by removal of the endothelium that ablated the response to 10 μmol/L carbachol (Figure S4). The response to (S)-1 was the same in all branches of the LAD coronary arteries (Figure S4C). The K\textsubscript{7}7.1 activator R-L3, recently shown to relax mesenteric and pulmonary arteries, had no effect on precontracted coronary arteries in a concentration range of 1 to 10 μmol/L (Figure 2D). The vasorelaxant effects of the K\textsubscript{7}7.2 to 7.5 activators were absent in coronary arteries isolated from SHRs (Figure 2C and 2D) although they relaxed fully on application of the Ca\textsuperscript{2+}-channel blocker nicardipine (10 nmol/L) or the ATP-sensitive K\textsuperscript{+} channel activator pinacidil (10 μmol/L; Figure S5). These data indicate that K\textsubscript{7}7 channels are active regulators of coronary arteries from normotensive but not hypertensive rats.

K\textsubscript{7}7 Channels in Coronary Artery Regulation

Quantitative PCR experiments showed that coronary arteries expressed KCNQ1, 4, and 5 with negligible contribution from KCNQ2 or 3 (Figure 3A). Similar to previous work, we found that LAD coronary arteries from SHRs exhibited considerably less K\textsubscript{7}7.4 protein compared with arteries from

Figure 1. Effect of selective pharmacological tools used to inhibit the function of K\textsubscript{7}7 channels in nonprecontracted coronary arteries. Isometric tension responses to cumulative application of K\textsubscript{7}7 channel inhibitor as compared with control (dimethyl sulfoxide [DMSO]) in normotensive (NT) and hypertensive (HT) rats. Representative traces of 1, 3, 5, and 10 μmol/L XE-991 (A) and linopirdine (B) in coronary arteries of NT and HT rats. Concentration-dependent effect curve of K\textsubscript{7}7 inhibitor XE-991 and linopirdine (C) in NT and spontaneously HT rats on arterial tone. Data are means±SEM. A 2-way ANOVA followed by a Bonferroni post-test was used. §§§P<0.001 for differences between the effect of the (S)-1 inhibitor XE-991 or linopirdine in NT rats compared with vehicle; ***P<0.001 for differences between the effect of the K\textsubscript{7}7 inhibitor XE-991 or linopirdine in NT compared with HT rats.
normotensive rats (Figure 3B). However, this was not because of a change in transcript level (Figure 3C) although KCNQ1 and KCNQ4 transcripts were decreased =6-fold in rat aorta in line with previous work (Figure 3D-i).a Mesenteric arteries also showed no change in KCNQ transcription (Figure 3D-ii). Coronary arteries also expressed all members of the KCNE gene family except KCNE1 (Figure 3E-i), and the expression of these genes was unchanged in arteries from SHRs (Figure 3E-ii).

Role of K<sub>v</sub>7 Channels in Vasodilatory Response to Adenosine

Experiments were performed to ascertain the contribution of K<sub>v</sub>7 channels to the physiological responses to adenosine. Both adenosine and the A2 receptor-selective agonist CGS-21680 relaxed precontracted coronary arteries by 25% to 30%, which was abrogated by prior incubation with 10 μmol/L linopirdine (Figure 4). Adenosine-mediated relaxations were also considerably less in coronary arteries from SHRs (Figure 4B). Consistent with this intracellular signaling pathway, the cell permeable cAMP analogue 8-Br-cAMP also produced concentration-dependent relaxations that were sensitive to linopirdine pretreatment (Figure S6A). To investigate whether protein kinase A activity is involved in regulating K<sub>v</sub>7 activity by adenosine, we incubated normotensive coronary arteries with the protein kinase A inhibitors KT5720 or H89 for 1 hour. Inhibition of protein kinase A fully antagonized the dilative response to adenosine (Figure S6B). Application of isoproterenol resulted in linopirdine-sensitive vasorelaxations that were less pronounced in SHR arteries (Figure S7), whereas the NO donor sodium nitroprusside produced relaxations that were not affected by K<sub>v</sub>7 blockade (Figure S8).

Isolated Perfused Heart Preparations

Ex vivo experiments were performed to determine the impact of K<sub>v</sub>7 blockade on coronary blood flow under normal and stressed conditions (Figure 5). We applied XE-991 (3 μmol/L) or linopirdine (10 μmol/L) in isolated, perfused heart preparations. Prolonged infusion with XE-991 or linopirdine to the isolated hearts from normotensive rats produced a decrease in coronary blood flow compared with DMSO-perfused hearts (Figure 5A). Coronary K<sub>v</sub>7 channels were efficiently blocked by the used concentrations as evidenced by a significantly diminished effect of (S)-1 (10 μmol/L) on coronary flow, when coinfused with either XE-991 or linopirdine (Figure 5B). Having established that K<sub>v</sub>7 channels contribute to coronary flow under resting conditions, we tested whether pharmacological blockade of K<sub>v</sub>7 channels had any effect on mechanisms regulating coronary flow, such as autoregulation, tachycardia-induced vasodilation, and reactive hyperemia. Infusion of a K<sub>v</sub>7 channel blocker to the heart had effect neither on coronary autoregulatory function nor on tachycardia-induced vasodilation (Figure 5C and 5D). In contrast, coronary reactive hyperemic responses after

**Figure 2.** Effect of accumulative application of K<sub>v</sub>7 channels activators on arterial coronary tone from normotensive (NT) rats and spontaneously hypertensive rats (SHRs) preconstricted with 1 nmol/L U46619. A, Representative trace of concentration-dependent relaxation to 1, 3, 10, and 30 μmol/L K<sub>v</sub>7 channel activator (S)-1 in the presence or absence of 10 μmol/L K<sub>v</sub>7 inhibitor linopirdine. B, Concentration–effect curve for 3 structurally different K<sub>v</sub>7 channel activators ((S)-1, BMS-204352, and retigabine) on coronary arteries. Each point is the mean of 11 to 22 NT coronary arteries±SEM. A 2-way ANOVA followed by a Bonferroni post-test was used as statistical analysis. *P<0.05; **P<0.01; and ***P<0.001 for differences between K<sub>v</sub>7 activators and control. C, Representative concentration–effect curve for KV7 channel activator (S)-1 in coronary arteries of NT and hypertensive (HT) rats. D, Comparison of KV7 channel activator effects on the coronary vasculature of NT and HT rats on preconstriction. Each point is the mean of 9 to 22 SHRs and 11 to 22 NT coronary arteries±SEM. *P<0.001, significance of effects of (S)-1, BMS-204352, and retigabine in SHRs compared with NT rats (2-way ANOVA followed by a Bonferroni post-test).
90 s of global ischemia were lower on K\textsubscript{v7} channel blockade (Figure 6A). A clearly attenuated level of reactive hyperemia was also observed in hearts from SHRs. As excess hyperemic flow depends on the preocclusion flow rate, the flow debt (preocclusion flow multiplied by ischemia duration) is lower in the hearts infused with a KV7 blocker (Table S2). Nevertheless, the percentage of flow repayment, which reflects the total flow at reperfusion normalized to flow debt, was still decreased in hearts with blocked KV7 channels. In addition, the hyperemic peak flow and total duration of reactive hyperemia were reduced on XE-991 or linopirdine infusion (Table S2). Overall, the data indicate that coronary KV7 channels contribute to the restoration of cardiac reperfusion after transient coronary occlusion.

**Discussion**

K\textsubscript{v7} channels are important regulators of vascular tone in several arteries, including mesenteric, renal, and cerebral arteries\textsuperscript{4,11} where K\textsubscript{v7} blockers promote vasospasm, and K\textsubscript{v7} activators are effective vasorelaxants\textsuperscript{4,6,7,11,14,15}. Moreover, evidence is accumulating that activation of K\textsubscript{v7} channels contributes to the vasodilatory response of various endogenous molecules, including \beta\textsubscript{2}-adrenoceptor agonists\textsuperscript{3} and H\textsubscript{2}S\textsuperscript{16}. In addition, mesenteric and renal arteries from hypertensive animals exhibit considerable attenuated response to KV7 activators and marked reduction in K\textsubscript{v7}.4 abundance\textsuperscript{6,14}. The present study now reveals that coronary arteries express KCNQ1–5 and KCNE1–5 genes in left anterior descending coronary arteries of normotensive (NT) rats and spontaneously hypertensive rats (SHRs). Quantitative polymerase chain reaction (PCR) analysis of NT rat KCNQ1–5 (n=3-6; A). Comparison of K\textsubscript{v7.4} protein expression (n=6-9; B) and individual KCNQ1–5 genes (n=3–6; C) in NT and hypertensive (HT) rats coronary arteries using \(2^{-\Delta\Delta CT}\) method. Comparison of KCNQ1–5 expression in NT and HT rats in thoracic aorta (D-i) and mesenteric arteries (D-ii). Quantitative PCR experiments of the auxiliary subunits KCNE1–5 coronary arteries of NT rats (E-i) and individual comparison of NT vs SHR rats (E-ii) using \(2^{-\Delta\Delta CT}\) method.
Hypertension was markedly impaired in coronary arteries from SHRs similar to findings in aorta, mesenteric and renal arteries, which was associated with a reduction in Kv7.4 protein abundance. In contrast to the rat aorta, there was no change in KCNQ expression in the coronary arteries or mesenteric arteries (also observed in gracilis muscle arteries from SHRs). This suggests that the decrease in Kv7.4 reflects an alteration in protein handling rather than transcriptional control. It is worth stressing that we detected an even greater reduction in KCNQ1 and KCNQ4 transcripts in the aorta compared with our previous study because we used a more rigorous quantification technique based around the geNorm analysis of stable housekeeping genes.

The present work also showed that Kv7 blockers attenuated basal coronary blood flow and reduced all indices of reactive hyperemia in normotensive rats after brief cessation of

Figure 4. Kv7 channel contribution to adenosine-mediated vasodilation. A, Representative traces of isometric tension recordings to concentration-dependent adenosine relaxation in coronary arteries in the presence and absence of 10 µmol/L Kv7 inhibitor linopirdine. B, Concentration–effect curve of adenosine in normotensive (NT) rats was compared with dimethyl sulfoxide (DMSO) control. Same was done for spontaneously hypertensive (HT) rats (data not shown). Each point is mean of 11 to 12 vessels±SEM. Comparison of vasorelaxations to adenosine in the presence and absence of linopirdine compared with DMSO control is illustrated by ***P<0.001. Comparison of concentration-dependent responses to adenosine between NT and HT coronary arteries is significant by §§§P<0.001 using a 2-way ANOVA followed by Bonferroni post-test. C, Concentration–effect curve for adenosine receptor 2A in coronary arteries of NT rats in the presence and absence of linopirdine as compared with its DMSO control. Each point is mean of 5 rat vessels±SEM and ***P<0.001 is significance between relaxations to CGS-21680 and DMSO by 2-way ANOVA and a Bonferroni post-test.

Figure 5. Effect of Kv7 blockers on basal coronary flow of normotensive (NT) perfused hearts. A, Fractional change of coronary flow vs baseline on 30 and 60 minutes infusion of dimethyl sulfoxide (DMSO) or a Kv7 channel blocker. B, Fractional change of coronary flow rate vs baseline on infusion of 10 µmol/L (S)-1 and 10 µmol/L (S)-1 with either 3 µmol/L XE-991 or 10 µmol/L linopirdine. C, Coronary flow as a function of pacing frequency in the hearts infused with DMSO and Kv7. D, Representative coronary flow trace in response to an increase in pacing frequency of an isolated heart perfused with DMSO. Data are mean±SEM. Unpaired t test and 1-way ANOVA test followed by Tukey’s multiple comparison post-test are used in A and B, respectively, and *P<0.05.
perfusion as well as abrogating the response to adenosine and the A2A receptor–specific agonist CGS-21680. Adenosine is a key mediator of coronary ischemic vasodilatation, which contributes to amelioration of reperfusion injuries.17–20 Levels of this molecule increase ≈3-fold within 5 s of myocardial ischemia in vivo,17 and the increase in coronary flow correlates highly with the rapid release of endogenous adenosine21–24 and the activation of the adenosine 2A receptor.13,25–28 Pharmacological blockade of adenosine receptors or limiting myocardial adenosine concentration during hypoxia and reperfusion results in diminished repayment flow and peak hyperemic flow in various animal species both in vivo and in isolated, perfused hearts.18,29–31

Activation of adenosine receptors leads to increased intracellular cAMP and possible stimulation of protein kinase A, but little is known about the downstream signals although recruitment of ATP-sensitive K channels has been implicated in porcine but not human coronary arteries.25 We recently proposed that the β-adrenoceptor agonist isoproterenol relaxed renal arteries through a cAMP-dependent recruitment of K,7 channels and K,7.4, in particular.14 Our pharmacological modulation of adenosine and isoproterenol-mediated responses with K,7 blockers and the poor response in arteries from SHRs suggests that a similar paradigm exists in the coronary circulation. As such, K,7 channels provide a crucial functional end point for adenosine-mediated signaling. In agreement with a key role for K,7 channels in coronary perfusion and reactive hyperemia, we show that K,7 blockade has a similar effect on all parameters of the coronary reperfusion (ie, duration, magnitude, flow repayment, and debt) as produced by adenosine antagonists. Our data provide an important insight into the molecular mechanisms that underlie key physiological mechanisms in coronary circulation and implicate K,7 channels as therapeutic targets in coronary ischemia. Moreover, the identification that K,7.4 becomes dysfunctional in hypertension provides a mechanism for coronary artery underperfusion and compromised reactive hyperemia in hypertensive individuals although changes in adenosine receptor number and other factors may also occur. Future studies will strive to determine the factors that dictate K,7.4 abundance.

**Perspectives**

In the present study, we show that K,7 channels are expressed in rat coronary arteries. We provide evidence that they are important for regulating coronary artery contractility and
coronary flow. Furthermore, using pharmacological tools we demonstrate that K\(_7\) channels have a central role in adenosine regulation of coronary artery contractility. Interestingly, in tissue from spontaneously hypertensive rats, the vasodilatory effect of adenosine was abolished. In line with this, we found a reduced K\(_7\) protein expression in hypertensive animals.

Adenosine levels increase on myocardial ischemia. Consequently, the release of adenosine is important for the hyperemic response that is the transient increase in blood flow after brief periods of ischemia. We found that K\(_7\) channels are involved in the reactive hyperemic response as inhibition of the channels attenuated the response. Overall, our findings provide evidence for a functional role of K\(_7\) channels in the coronary circulation under physiological conditions and after ischemic insults. Noteworthy, the vasodilatory potential of K\(_7\) in the coronary arteries was diminished in the hypertensive rat. Therapeutic strategies for improving K\(_7\) function could be of potential interest in clinical settings of reduced coronary flow.

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Disclosures
None.

References


### Novelty and Significance

**What Is New?**
- $K_v7$ channels are expressed in rat coronary arteries.
- $K_v7$ channels have a central role in adenosine regulation of coronary artery contractility.
- $K_v7$ channels are involved in the reactive hyperemic response as inhibition of the channels attenuates the response.

**What Is Relevant?**
- In tissue from spontaneously hypertensive rats, the vasodilatory effect of adenosine was abolished coinciding with reduced $K_v7$ protein expression in hypertensive animals.

**Summary**

Determining what regulates coronary blood flow is crucial for understanding the pathophysiology of coronary artery disease and in the development of new therapeutic strategies. Our studies add $K_v7.4$ and $K_v7.5$ channels as possible candidates to the equation.

- Therapeutic strategies for improving $K_v7$ function could be of potential interest in clinical settings of reduced coronary flow.
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Contribution of $K_v$7 channels to basal coronary flow and active response to ischemia

Short title: $K_v$7 channels in coronary artery regulation

Saereh Khanamiri$^1$, Ewa Soltysinska$^1$, Thomas A. Jepps$^2$, Bo H. Bentzen$^1$, Preet S. Chadha$^2$, Nicole Schmitt$^{1,*}$, Iain A. Greenwood$^2$, Søren-Peter Olesen$^1$

$^1$Danish National Research Foundation Centre for Cardiac Arrhythmia and Dept. of Biomedical Sciences, University of Copenhagen, Denmark

$^2$Pharmacology & Cell Physiology Research Group, Division of Biomedical Sciences, St. George’s University of London, London, UK

*Corresponding author:
Nicole Schmitt, PhD
Danish National Research Foundation Centre for Cardiac Arrhythmia and Dept. of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen
The Panum Institute, 12.5.14, Blegdamsvej 3, 2200 Copenhagen N, Denmark
Phone: +45 35327448, Fax: +45 35327555, E-mail: nschmitt@sund.ku.dk
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SUPPLEMENTAL METHODS

Isometric Tension Recordings
First, second and third order branches of LAD coronary arteries from normotensive and hypertensive rats were carefully dissected and transferred to ice-cold Krebs Solution (in mmol/L: 133 NaCl, 4.6 KCl, 2.5 CaCl$_2$, 16.3 NaHCO$_3$, 1.75 Na$_2$HPO$_4$, 0.6 MgSO$_4$, 10 glucose) and cleaned from adherent connective tissue. These vessels were threaded on 40 μm tungsten wires and mounted in an isometric wire myograph chamber (DanishMyo Technology, Aarhus, Denmark) containing Krebs solution constantly aerated with 95% O$_2$/5% CO$_2$ at 37°C. All vessels were subjected to a standard warm-up protocol where they were repeatedly stimulated with 60 mmol/L KCl. PowerLab4/25-Chart5 and 7 acquisition systems (ADInstruments Ltd, Oxford, UK) were used to record force which was converted to tension by dividing the force with double the segment length. By cumulative addition of the synthetic analog of prostaglandin U46619 within a concentration range of 0.1-10 nmol/L a stable vessel contraction corresponding to the same contraction achieved by applying 60-100 nmol/L KCl was determined to be 1nmol/L U46619.

Subsequently concentration-response relationship curves of K$_v$7 activators ((S)-1, BMS-254352, retigabine (1-30 μmol/L) and R-L3 (1-10 μmol/L), β-adrenoceptor isoproterenol (1-30 μmol/L), the NO donor SNP (0.5-10 μmol/L) and the metabolic substrates of adenosine (1-30 μmol/L) were established by cumulative addition of drugs in presence and absence of linopirdine coronary arteries from both normotensive and hypertensive SHR rats. Effects of K$_v$7 blockers on resting tone were addressed by cumulative addition of linopirdine, XE-991 (1-10 μmol/L) or HMR-1556 without use of U46619. Investigation of protein kinase A activity in normotensive vessels was performed by incubation of coronary arteries with 500 nmol/L PKA inhibitors KT5720 or H89. Dimethylsulfoxide (DMSO) was used as control in all experiments.

Langendorff-Perfused Hearts
Rats were anesthetized and anticoagulated by intraperitoneal injection of heparin (1000 UI/kg). The hearts were excised, placed in ice-cold perfusion solution and mounted on a perfusion system (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) and perfused in the Langendorff mode at constant perfusion pressure (80 mmHg) with warm (37°C) modified Krebs-Henseleit (KH) solution (in mmol/L: 118.0 NaCl, 4.7 KCl, 2 CaCl$_2$, 1.2 KH$_2$PO$_4$, 1.2 Mg$_2$SO$_4$, 2.0 sodium pyruvate, 24.9 NaHCO$_3$, and 10.0 glucose, pH of 7.4–7.5). KH solution was continuously aerated with 95 % O$_2$/5 % CO$_2$ and was filtered through an in-line 0.45 μm Sterivex-HV filter (Millipore A/S, Hellerup, Denmark) before delivery to the heart. Coronary flow was monitored via an ultrasonic flow meter probe (Transonic Systems Inc., Ithaca, USA) located above the aortic cannula. Electrical activity of the heart was assessed from volume-conducted ECG, recorded via four Ag/AgCl electrodes placed in the perfusate-filled chamber around the heart preparation. Aortic pressure, coronary flow, ECG and perfusate temperature were continuously monitored using the 16-channel Powerlab system (ADInstruments, Oxford, UK) and recorded by LabChart 7 software. All pharmacological agents ((S)-1, XE-991, linopirdine) and DMSO were infused to the heart via perfusate. Subsequently, the effect of blocking K$_v$7 channels on tachycardia-induced vasodilatation was investigated by increasing the pacing frequency from basic rate of 380 to 440, 500 and 600 bpm at 150 second intervals. Reactive hyperemic responses were induced by 30 seconds and 90 seconds of no-flow to the cannulated aorta and thereby the coronary arteries, global ischemia periods, followed by at least 8-9 minutes of reperfusion at which coronary flow returned to the pre-occlusion levels. The following
indices of reactive hyperemic responses were assessed: basal flow rate (mL/min) [mean flow rate over 2 min pre-occlusion], flow debt (mL) [basal flow rate (mL/min) multiplied by ischemia duration (min)], excess hyperemic flow (mL) [integral of flow over initial 5 min of reperfusion (mL) minus integral of flow over 5 min pre-occlusion (mL)], flow repayment (%) [excess hyperemic flow (mL) x 100% divided by the flow debt (mL)], duration of reactive hyperemia (s) [time span from the start of reperfusion to the return of the flow rate to the level 5% higher than basal value], peak hyperemic flow rate (mL/min) [maximal flow at reperfusion].

**Quantitative Polymerase Chain Reaction**

Total RNA was extracted from first order LAD coronary arteries from normotensive and hypertensive rats using the RNeasy Micro Kit (Qiagen, Manchester, UK) as described. RNA was quantified using a Nanodrop Spectrophotometer (LabTech International, Carlson City, U.S) and reverse transcribed with Oligo(dT)12-18 primers and Moloney Murine Leukemia Virus (M-MLV; Invitrogen, Paisley, UK). Negative controls (RT-) were carried out in the absence of M-MLV to check for genomic contamination. Quantitative mRNA analysis was determined in duplicate reactions of 10 µL volumes using Precision-iC SYBR green master mix (PrimerDesign, Ltd., Southampton, UK) with the CFX96™ Real-Time PCR Detection System (Biorad, Hertfordshire, UK). Cycle threshold (Ct) values were determined using Bio-Rad CFX96 software and the single threshold mode. To determine the optimal reference genes we used the rat geNorm Reference Gene Selection Kit (PrimerDesign, Ltd., UK), consisting of 12 commonly used reference genes, which were run on normotensive rat (n=3-6) and SHR (n=3-6) LAD coronary artery cDNA samples. The data were then analyzed with geNorm software to determine the best reference gene(s) and number of reference genes required for the most accurate gene normalization (Vandesompele et al., 2002). For the coronary artery experiments the optimal number of reference genes was 2 (where geNorm V < 0.15): β-Actin and GAPDH. In the aorta the reference genes used were CYC1 and MDH1, and in the mesenteric artery ATP5B and YWHAZ were used as reference genes, determined under our experimental conditions as the most stable in the given samples (PrimerDesign, Ltd., UK). No template controls (NTCs) were run alongside all reactions to assess contamination. To determine the change in KCNQ1-5 and KCNE1-5 expression between normotensive rat and SHR arteries, the 2^ΔΔCt method was used (Livak and Schmittgen, 2001). Primer sequences are listed in Suppl. Table S1.

**Western Blot Analysis**

LAD coronary arteries from normotensive (n=3-9) and hypertensive rats (n=3-9) were homogenized in 200 µL lysis buffer (2 mmol/L EDTA, 20 mmol/L Tris base, 1% NP40, 137 mmol/L NaCl, 10% glycerol, 10 µL/mL protease inhibitor cocktail; Sigma-Aldrich, Brøndby, Denmark), centrifuged to remove cell debris, and denatured at 95°C for 5 minutes in the presence of reducing agent and sample buffer (Invitrogen, Nærø, Denmark). Proteins were separated on 4-12% SDS-polyacrylamide gels (Invitrogen) and transferred to immunoblot PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 5% milk in PBS-Tween (0.1%) and incubated with the primary antibody against Kv7.4 (1 µg/mL; Santa Cruz, Santa Cruz, CA, USA). The membrane was washed, reprobed for β-actin (0.4 µg/mL; Sigma-Aldrich, A1978, Germany) and visualized by enhanced chemiluminescence (ECL) staining. All bands were normalized against β-actin.
SUPPLEMENTAL REFERENCES

## SUPPLEMENTAL TABLES AND FIGURES

### Supplemental Table S1: KCNQ and KCNE primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>GenBank Accession Number</th>
<th>Amplicon (bp)</th>
<th>Region Spanned (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNQ1</td>
<td>(+) 5'-CCATCTTTGTTCATCCCCATCT-3’ (-) 5’- CCAGTTGTCACCTTGTCTT -3’</td>
<td>NM_032073</td>
<td>100</td>
<td>1797-1896</td>
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<tr>
<td>KCNQ2</td>
<td>(+) 5’-GGTGTCATCTTCATTCTGCTCCTT-3’ (-) 5’-TCCGCCGTTTCTCAAAGTG-3’</td>
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<td>KCNQ3</td>
<td>(+) 5’-ATACACATTTATCTGCTCTTCTTTTA-3’ (-) 5’-TGCTCAGTTATCCGAAATCAA-3’</td>
<td>NM_031597</td>
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<td>KCNQ4</td>
<td>(+) 5’-GCTCATCTTCCTGCTCCTTTACC-3’ (-) 5’-GCCAATGGTGTCAGTGAAT-3’</td>
<td>XM_233477</td>
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<tr>
<td>KCNQ5</td>
<td>(+) 5’-CCTGGCCGTACACGGAGATAT-3’ (-) 5’-TTTGACTGGGCGAACTGAAC-3’</td>
<td>XM_001071249</td>
<td>80</td>
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<td>KCNE1</td>
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<td>KCNE3</td>
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<td>Gene</td>
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<td>(-) Sequence</td>
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<tr>
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<td>KCNE5</td>
<td>(+) 5’-GTCAACGGCGTCCTGGAG-3’</td>
<td>(-) 5’-CAGCAGCAAGCGGTTCAA-3’</td>
<td>NM_00110100</td>
<td>96</td>
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(+): sense, (-): antisense; bp: base pair, nt: nucleotide. Information on proprietary primers used in the geNorm Reference Gene Selection Kit is not available.
**Supplemental Table S2. Indices of coronary reactive hyperemia**

<table>
<thead>
<tr>
<th>Parameter/Treatment group</th>
<th>DMSO (n=8)</th>
<th>linopirdine / XE-991 (n=9)</th>
<th>SHR (n=5)</th>
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<tbody>
<tr>
<td>Basal flow rate (mL/min)</td>
<td>12.7±0.3</td>
<td>11.5±0.2,*</td>
<td>7.79±2.1,*</td>
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<tr>
<td>Flow debt (mL)</td>
<td>19.0±0.47</td>
<td>17.2±0.35,*</td>
<td>9.78±2.3,*</td>
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<tr>
<td>Excess hyperemic flow (mL)</td>
<td>19.8±1.8</td>
<td>13.5±0.9,*</td>
<td>0.97±2.4,*</td>
</tr>
<tr>
<td>Flow repayment (%)</td>
<td>105.7±11.6</td>
<td>78.8±5.3,*</td>
<td>14.5±8.8,*</td>
</tr>
<tr>
<td>Duration of reactive hyperemia (s)</td>
<td>324.6±31.9</td>
<td>219.9±18.0,*</td>
<td>187.6±8.0,*</td>
</tr>
<tr>
<td>Peak hyperemic flow rate (mL/min)</td>
<td>30.2±0.3</td>
<td>27.4±0.7,*</td>
<td>19.4±1,*</td>
</tr>
</tbody>
</table>

Reactive hyperemia in isolated, perfused NT hearts infused either with DMSO or a K\textsubscript{v}7 blocker and reactive hyperemia in SHRs as compared to its control. Data are mean ± SEM. An un-paired t-test is used for the comparison and * indicates $P<0.05$. 

Supplemental Figure S1: Diameter size and force of first, second and third order LAD branches in normotensive rats and comparison of basal force between normotensive and hypertensive LAD. No differences were found in diameter size (A) and basal force (B) of first, second and third order LAD coronary artery branches in normotensive rats upon normalization. No differences were found in basal force in comparison of normotensive and hypertensive arteries (C). An one-way ANOVA test was used in the panel A-B and an unpaired t-test in the panel C.
Supplemental Figure S2. Effect of HMR-1556 used to specifically inhibit the function of Kv7.1 channels in non-pre-constricted coronary arteries. (A) Isometric tension responses to accumulative application of HMR-1556 in a concentration range of 1-10 μmol/L as compared to dimethyl sulfoxide (DMSO) control vehicle in normotensive rats. Statistical significance was found using a two-way ANOVA followed by a Bonferroni post-test between HMR and DMSO vehicle group, **P<0.01 and ***P<0.001.
Supplemental Figure S3. Contraction to high $K^+$ concentrations and prostaglandin agonist U46619 in normotensive and hypertensive rats. (A) Comparison of isometric tension responses to 60 mmol/L KCl and 1nmol/L U46619 in LAD coronary arteries of normotensive and hypertensive rats. (B) Dose-dependent responses to cumulative application of the prostaglandin agonist U46619 in normotensive and hypertensive SHR vasculature.
Supplemental Figure S4. Relaxation to K\textsubscript{\textgamma}7 activator in vessels with intact and denuded endothelium and in different branch orders of LAD arteries in normotensive rats. (A) Relaxation to 10 \textmu mol/L Carbachol was significantly impaired in denuded coronary arteries as compared to LAD vessels with intact endothelium. (B) Relaxation to (S)-1 was comparable between vessels with intact endothelium and endothelium-denuded LAD coronary arteries. (C) No significant dilative response differences were when comparing the effect of (S)-1 in first, second and third order branches of LAD coronary artery from normotensive rats. Statistical analysis was performed by two-way ANOVA test.
Supplemental Figure S5. Relaxation to nicardipine and pinacidil in normotensive and hypertensive rats. Both nicardipine (10 nmol/L) (A) or pinacidil (10 µmol/L) (B) had a vasodilatory effect on preconstricted vessels of both normotensive and hypertensive vessels. No differences in percentage relaxation were found between normotensive and hypertensive arteries as assessed with unpaired t-test. (C) illustrates the vasorelaxant effect of 10 µmol/L K\textsubscript{ATP} opener pinacidil in presence of 10 µmol/l K\textsubscript{V7} channel blocker linopirdine on preconstricted LAD coronary arteries of normotensive rats. Similarly, the K\textsubscript{V7} activator (S)-1 has a vasorelaxant effect on preconstricted coronary arteries from normotensive rats in the presence of the K\textsubscript{ATP} channel blocker glibenclamide. Significant difference were found by *** p<0.001 by ANOVA Bonferroni post-test which was done on mean values of 4-9 vessels ±SEM.
Supplemental Figure S6. Effect of PKA activation and inhibition in LAD of normotensive rats. (A) Concentration-dependent effects of adenosine 2A receptor agonist, cyclic AMP analog 8-Br-cAMP, on normotensive coronary arteries. (B) Concentration-dependent effects of adenosine on LAD of normotensive rats upon incubation with PKA inhibitors KT5720 or H89 (1 μmol/L) for 1 hour. Significant difference in responses to 8-Br-cAMP in presence and absence of 10 μmol/L linopirdine upon incubation with PKA inhibitors by ***p<0.001 by ANOVA Bonferroni post-test which was done on mean values of 9 vessels ±SEM.
A. % Relaxation of initial contraction vs. [Isoproterenol] (μmol/L) for NT and NT + 10 μmol/L linopirdine. 

B. % Relaxation of initial contraction vs. [Isoproterenol] (μmol/L) for NT and HT. 

C. % Relaxation of initial contraction vs. [Isoproterenol] (μmol/L) for NT and NT + 60 min in 1 μmol/L KT5720 or H89. 

n=10-11

n=8-9
Supplemental Figure S7. Isometric tension responses of coronary arteries to β-adrenergic stimulation. (A) Application of isoproterenol resulted in vasodilation of coronary arteries in normotensive rats. The vasodilatory effect was significantly attenuated upon linopirdine. (B) However, coronary arteries of hypertensive SHR rats were resistant to isoproterenol-mediated vasorelaxation. (C) The same attenuated effect of isoproterenol was found in normotensive coronary arteries upon PKA inhibition. Two-way ANOVA and a Bonferroni post-test was used, * P<0.05, ** P<0.01 and *** P<0.001.
Supplemental Figure S8. Isometric tension responses of coronary arteries to NO-donor SNP. Application of SNP resulted in vasodilation in normotensive rats and this effect was not changed upon linopirdine application. Vasodilation to 10 and 30 μmol/L SNP was attenuated in hypertensive as compared to normotensive vasculature. PKA inhibition had no effect on vasodilatory responses to SNP in normotensive vasculature. Two-way ANOVA and a Bonferroni post-test was used and § indicated $P<0.05$, ** $P<0.01$ and *** $P<0.001$