British Journal of Pharmacology (2007) 152, 1060–1069
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Protein kinase G regulates the basal tension and plays a major role in nitrovasodilator-induced relaxation of porcine coronary veins

H Qi[1], X Zheng[1], X Qin[1], D Dou[1], H Xu[1], JU Raj[2] and Y Gao[1,3]

[1]*Department of Physiology and Pathophysiology, Peking University Health Science Center, Beijing, China;* [2]*Division of Neonatology, Harbor-UCLA Medical Center, University of California at Los Angeles Geffen School of Medicine, Los Angeles, CA, USA and* [3]*Key Laboratory of Molecular Cardiovascular Sciences (Peking University), Ministry of Education, Beijing, China*

Background and purpose: Coronary venous activity is modulated by endogenous and exogenous nitrovasodilators. The present study was to determine the role of protein kinase G (PKG) in the regulation of the basal tension and nitrovasodilator-induced relaxation of coronary veins.
Experimental approach: Effects of a PKG inhibitor on the basal tension and responses induced by nitroglycerin, DETA NONOate, and 8-Br-cGMP in isolated porcine coronary veins were determined. Cyclic cGMP was measured with radioimmunoassay. PKG activity was determined by measuring the incorporation of ^{32}P from γ-^{32}P-ATP into the specific substrate BPDEtide.
Key results: Rp-8-Br-PET-cGMPS, a specific PKG inhibitor, increased the basal tension of porcine coronary veins and decreased PKG activity. The increase in tension was 38% of that caused by nitro-L-arginine. Relaxation of the veins induced by nitroglycerin and DETA NONOate was accompanied with increases in cGMP content and PKG activity. These effects were largely eliminated by inhibiting soluble guanylyl cyclase with ODQ. The increase in PKG activity induced by the nitrovasodilators was abolished by Rp-8-Br-PET-cGMPS. The relaxation caused by these dilators and by 8-Br-cGMP at their EC_{50} was attenuated by the PKG inhibitor by 51–66%.
Conclusions and implications: These results suggest that PKG is critically involved in nitric oxide-mediated regulation of the basal tension in porcine coronary veins and that it plays a primary role in relaxation induced by nitrovasodilators. Since nitric oxide plays a key role in modulating coronary venous activity, augmentation of PKG may be a therapeutic target for improving coronary blood flow.
British Journal of Pharmacology (2007) **152,** 1060–1069; doi:10.1038/sj.bjp.0707479; published online 24 September 2007

Keywords: nitric oxide; Rp-8-Br-PET-cGMPS; myristoylated PKI

Abbreviations: 8-Br-cAMP, 8-bromo-adenosine 3′,5′-cyclic monophosphate; 8-Br-cGMP, 8-bromo-guanosine 3′,5′-cyclic monophosphate; EDNO, endothelium-derived nitric oxide; ODQ, 1H-[1,24]oxadiazolo[4,3-a]quinoxalin-1-one; Rp-8-Br-PET-cGMPS, β-phenyl-1, N^2-etheno-8-bromoguanosine-3′5′-cyclic monophosphorothioate, Rp-isomer

Introduction

The coronary venous system has long been considered a passive conduit from which coronary blood flows into the right atrium. There is accumulating evidence to show that coronary veins exhibit potent contractile response to vasoconstrictors such as noradrenaline, 5-HT, U46619, endothelin-1 and KCl (Chilian *at al*., 1989; Cocks *et al*., 1989a, b; Gulbenkian *et al*., 1994; Saetrum Opgaard *et al*., 1996; Saetrum Opgaard and Edvinsson, 1997; Zhang *et al*., 2004) and marked relaxant response to vasodilators such as sodium nitroprusside, isoprenaline, adenosine and vaso-active intestinal peptide (Gulbenkian *et al*., 1994; Banitt *et al*., 1995; Saetrum Opgaard *et al*., 1995; Saetrum Opgaard and Edvinsson, 1997). Canine coronary veins demonstrate 5- to 10-fold greater sensitivity to endothelin-1 than the coronary arteries (Cocks *et al*., 1989a, b). In humans, noradrenaline and ATP cause greater contraction of coronary veins than that of coronary arteries (Saetrum Opgaard and Edvinsson, 1997). These venous responses may contribute importantly to the regulation of myocardial blood flow and fluid filtration under physiological and pathophysiological conditions (Tiefenbacher and Chilian, 1998; Tune *et al*., 2004).

Correspondence: Dr Y Gao, Department of Physiology and Pathophysiology, Peking University Health Science Center, 38 Xue Yuan Road, Haidian District, Beijing 100083, China.
E-mail: ygao@bjmu.edu.cn
Received 9 July 2007; revised 14 August 2007; accepted 29 August 2007; published online 24 September 2007

Endothelium-derived nitric oxide (EDNO) plays a pivotal role in the regulation of the coronary circulation (Drexler, 1999). In porcine coronary veins, EDNO is released under basal conditions and in response to bradykinin (Zhang *et al*., 2004). Both *in vitro* and *in vivo* studies show that porcine coronary venules dilate in response to flow stimuli in an EDNO-dependent manner. Such a phenomenon may contribute to the adjustment of postcapillary resistance to maintain optimal myocardial perfusion and fluid filtration across the capillary wall during cardiovascular stress, such as physical exercise. However, when the veins are denuded of endothelium, the dilation induced by flow is converted to constriction, suggesting that when the vascular endothelium is damaged, blood flow and oxygen transport to the heart may be compromised during intense metabolic demands (Kuo *et al*., 1993).

In various vessel types including coronary arteries, EDNO and nitric oxide-releasing agents may relax the vessels through activation of soluble guanylyl cyclase, followed by elevation of cGMP and activation of cGMP-dependent protein kinase (PKG) (Francis and Corbin, 1994; Walsh *et al*. 1995; Lohmann *et al*., 1997; Gao *et al*., 1999; Lincoln *et al*., 2001; Qin *et al*., 2007). However, nitric oxide may also cause relaxation through cGMP-independent as well as PKG-independent mechanisms (Abderrahmane *et al*., 1998; Janssen *et al*., 2000; Francis *et al*., 2005). The contribution of PKG to the EDNO-mediated regulation of the basal tension and of responses induced by nitrovasodilators is not well understood. In coronary veins, although EDNO may be critically involved in the responses to various stimuli, the role of PKG has not been studied. By using selective inhibitors of PKG and by combining vessel tension studies with measurements of cGMP and PKG activity, this study demonstrates that PKG is importantly involved in nitric oxide-mediated regulation of basal tension of porcine coronary veins and that it plays a primary role in relaxation induced by nitroglycerin and nitric oxide.

Methods

Organ chamber study

Fresh pig hearts were obtained from a local abattoir. Middle cardiac veins (outside diameter: 1.79 ± 0.42 mm, $n = 27$) were dissected free and placed into ice-cold modified Krebs–Ringer bicarbonate solution (composition (in mM): NaCl, 118.3; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.1). Vessel rings were suspended in organ chambers filled with 10 ml Krebs–Ringer bicarbonate solution at 37 °C and aerated with 95% O_2–5% CO_2 (pH 7.4). Each ring was suspended by two stirrups passed through the lumen. One stirrup was anchored to the bottom of the organ chamber; the other one connected to a strain gauge (PowerLab/8sp, ADI Instrument, Bella Vista, Australia) for the measurement of isometric force (Gao *et al*., 1995; Wang *et al*., 2006).

At the beginning of the experiment, the vessel rings were stretched to their optimal basal tension (0.89 ± 0.07 g, $n = 10$). This was achieved by stepwise increases in tension (0.2 g increment) until the contractile response to 100 mM KCl reached a plateau (2.13 ± 0.39 g, $n = 10$, $P < 0.05$). Then 1 h of equilibration was allowed. Studies suggest that, at the optimal basal tension, the vessel ring is likely to be stretched to its optimum length for muscle contraction, that is, a length at which actin/myosin intercalation is optimal for tension generation (Gordon *et al*., 1966).

Effects of nitroglycerin (10^{-9}–3×10^{-4} M), DETA NONOate (10^{-7}–10^{-4} M), 8-bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP; 10^{-7}–10^{-4} M; a cell membrane-permeable analogue of cGMP (Meyer and Miller, 1974)) and 8-bromo-adenosine 3′,5′-cyclic monophosphate (8-Br-cAMP; 10^{-7}–10^{-4} M; a cell membrane-permeable analogue of cAMP (Meyer and Miller, 1974)) were determined in vessels preconstricted with U46619 (10^{-7}–2×10^{-7} M), a thromboxane A_2 analogue (Coleman *et al*., 1981) to a similar tension. The responses to the above nitrovasodilators and the nucleotide analogues were performed in the presence of indomethacin (10^{-5} M) plus nitro-L-arginine (10^{-4} M) to prevent the involvement of endogenous prostaglandins and nitric oxide (Cassin, 1980; Mülsch and Busse, 1990; Gao *et al*., 1996). Indomethacin and nitro-L-arginine did not significantly affect relaxation of the veins to DETA NONOate (data not shown, $n = 4$–6, $P > 0.05$).

In some experiments, the effects of various inhibitors on the basal tension and relaxant responses of the veins were examined. The experiments of control vessels and those of various treatments were conducted simultaneously. For each vessel ring, only one vasodilator was tested.

Radioimmunoassay of cGMP

Rings of porcine coronary veins with or without endothelium were incubated in modified Krebs–Ringer bicarbonate solution (37 °C, 95% O_2–5% CO_2). After 30 min of equilibration, solvent nitro-L-arginine (10^{-4} M) or 1H-[1,24]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 3×10^{-5} M) was added. Forty minutes later, isobutylmethylxanthine (10^{-3} M) was added. After another 30 min, solvent nitroglycerin (3×10^{-5} M) or DETA NONOate (3×10^{-5} M) was added, respectively. Vessel rings were snap-frozen 10 min later with liquid nitrogen, thawed in trichloroacetic acid (6%), homogenized in a glass mortar on ice, sonicated for 5 s and centrifuged (13 000 *g*) for 10 min. The supernatant was extracted with three volumes of water-saturated diethyl ether and lyophilized. The lyophilized samples were resuspended in 0.5 ml of sodium acetate buffer (0.05 M, pH 6.2) and the contents of cGMP were determined using cGMP kits (Department of Nuclear Medicine of Shanghai University of Traditional Chinese Medicine). The content of cGMP is expressed as pmol per mg protein of vessel homogenate. The protein content was determined based on the Bradford dye-binding procedure (Bradford, 1976).

PKG activity assay

For PKG activity assay of intact coronary veins, the vessels were first incubated in modified Krebs–Ringer bicarbonate solution containing indomethacin (10^{-5} M) and isobutylmethylxanthine (10^{-3} M) at 37 °C (95% O_2–5% CO_2) in the presence or absence of nitro-L-arginine (10^{-4} M), ODQ

(3×10^{-5} M) or β-phenyl-1, N^2-etheno-8-bromoguanosine-3′5′-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-PET-cGMPS; 3×10^{-5} M). After 40 min of incubation, the solvent nitroglycerin (3×10^{-5} M) or DETA NONOate (3×10^{-5} M) was added, 10 min later the tissue were snap-frozen with liquid nitrogen, homogenized in a buffer containing 50 mM Tris-HCl (pH 7.4 at 22 °C), 20 mM EDTA, 2 mM dithiothreitol, 1 mM isobutylmethylxanthine, 100 μM nitro-L-arginine and 10 μM indomethacin. The homogenate was sonicated and centrifuged at 13 000 *g* for 10 min at 4 °C. Protein content in supernatant was measured by Bradford's procedure, using bovine serum albumin as a standard (Bradford, 1976). Supernatants were assayed for PKG activity by measuring the incorporation of ^{32}P from γ-^{32}P-ATP into a specific PKG substrate BPDEtide (Alexis Corporation, San Diego, CA, USA), a peptide (RKISASEFDRPLR) derived from the sequence of the phosphorylation site in cGMP-binding cGMP-specific phosphodiesterase (Colbran *et al*., 1992).

The assay was started by adding aliquots (20 μl) of supernatant into a mixture (total volume, 50 μl) containing 50 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 1 mM isobutylmethylxanthine, 10 μM indomethacin, 100 μM nitro-L-arginine, 150 μM BPDEtide, 1 μM PKI (a synthetic PKA inhibitor; Biomol Research Laboratories, Plymouth Meeting, PA) and 0.2 mM γ-^{32}P-ATP (specific activity: 3000 Ci mmol^{-1}). The mixture was incubated at 30 °C for 10 min. Reaction was terminated by spotting 40 μl aliquots onto phosphocellulose papers (2 × 2 cm; P81 Whatman) and placed into ice-cold 75 mM phosphoric acid. The filter papers were washed, dried, and counted with a liquid scintillation counter. Assays were performed in triplicate with appropriate controls. PKG activity is expressed as pmol of ^{32}P-incorporated into PKG substrate min^{-1} mg^{-1} protein (Gao *et al*., 1999). Preliminary experiments confirmed the linearity of PKG activity at the protein concentration used within the incubation time.

For PKG activity assay of the homogenate preparations, the assay was conducted similar to that for intact vessels, except that vessels were not incubated prior to homogenization and the activity of PKG was stimulated with solvent or exogenous cGMP (3.16 μM) added in the assay buffer.

PKA activity assay

PKA activity assay was carried out in similar manner to that of PKG, excepting that PKG substrate was replaced with a specific PKA substrate, Kemptide (130 μM; Alexis Corporation), cGMP was replaced with cAMP (3.16. μM), and PKI was omitted. The linearity of PKA activity at the protein concentration used within the incubation time was determined in preliminary experiments.

Data analyses

Data are shown as means ± s.e. mean. When mean values of two groups were compared, Student's *t*-test for unpaired observations was used. When the mean values of the same group before and after stimulation were compared, Student's *t*-test for paired observations was used. Comparison of mean values of more than two groups was performed with one-way ANOVA test with Student–Newman–Keuls test for *post hoc* testing of multiple comparisons. Statistical significance was accepted when the *P*-value (two-tailed) was <0.05. In all experiments, *n* represents the number of animals.

Reagents

The following drugs were used (unless otherwise specified, all were obtained from Sigma, St Louis, MO, USA): 8-Br-cAMP and 8-Br-cGMP ; Biolog Life Science Institute, La Jolla, CA, USA), DETA NONOate (Cayman Chemical, Ann Arbor, MI, USA), indomethacin, myristoylated PKI (Biomol Research Laboratories, Plymouth Meeting, PA, USA), nitro-L-arginine, nitroglycerin (Beijing Yimin Pharmaceutical Co. Ltd, Beijing, China), ODQ, Rp-8-Br-PET-cGMPS (Biolog Life Science Institute, Bremen, Germany), U46619 (Alexis Biochemicals, San Diego, CA, USA).

ODQ was dissolved in DMSO (final concentrations: <0.2%). Preliminary experiments showed that DMSO at the concentration used had no effect on contraction to U46619 and relaxation induced by nitric oxide, nitroglycerin and 8-Br-cGMP in porcine coronary veins. Indomethacin (10^{-5} M) was prepared in equimolar Na_2CO_3. This concentration of Na_2CO_3 did not significantly affect the pH of the solution in the organ chamber. The other drugs were prepared using distilled water.

Results

Organ chamber studies

Under basal conditions, Rp-8-Br-PET-cGMPS, a specific inhibitor of PKG caused a concentration-dependent contraction of porcine coronary veins (Figure 1). The increase in tension caused by Rp-8-Br-PET-cGMPS at 3×10^{-5} M was 38 ± 9% of that caused by nitro-L-arginine (10^{-4} M; 0.28 ± 0.06 g, $P<0.05$), an inhibitor of nitric oxide synthase. The PKG inhibitor had no effect on the vessel tension in the presence of nitro-L-arginine (10^{-4} M) (Figure 1 and Table 1).

ODQ (3×10^{-5} M), an inhibitor of soluble guanylyl cyclase, increased the basal tension of the veins to about the same extent as nitro-L-arginine. In the presence of nitro-L-arginine, ODQ caused no further change in the tension. Myristoylated PKI (6×10^{-6} M), a cell-permeable inhibitor of cAMP-dependent protein kinase, PKA, caused a moderate increase in the basal tension (0.05 ± 0.02 g, $P<0.05$). The presence of myristoylated PKI had no significant effect on contraction induced by nitro-L-arginine (Table 1).

Relaxation induced by nitrovasodilators was determined in porcine coronary veins preconstricted with U46619 (a thromboxane A_2 analogue) to a similar level of tension (Table 2). Nitroglycerin and DETA NONOate caused a concentration-dependent relaxation which was largely abolished by ODQ (3×10^{-5} M; Figure 2). In the presence of Rp-8-Br-PET-cGMPS (3×10^{-5} M), the relaxation induced by nitroglycerin and DETA NONOate at EC_{50} was attenuated by 63 and 66%, respectively. The relaxation was not significantly affected by myristoylated PKI (6×10^{-6} M) (Figure 2).

8-Br-cGMP caused concentration-dependent relaxation of porcine coronary veins, which was more extensive than that caused by 8-Br-cAMP (Figure 3). The relaxation induced by

8-Br-cGMP at EC_{50} was inhibited by Rp-8-Br-PET-cGMPS (3×10^{-5} M) by 51% but was not significantly affected by myristoylated PKI (6×10^{-6} M). The relaxation of the veins to 8-Br-cAMP (3×10^{-5} M) was inhibited by myristoylated PKI by 59% but was not significantly affected by Rp-8-Br-PET-cGMPS (3×10^{-5} M) (Figure 3).

cGMP assay

As shown in Figure 4, under basal conditions, the intracellular content of cGMP of isolated porcine coronary veins with endothelium was greater than that of vessels without endothelium, vessels with endothelium treated with nitro-L-arginine or those treated with ODQ (Figure 4). In the veins without endothelium (lower half of Figure 4), nitroglycerin (3×10^{-5} M) and DETA NONOate (3×10^{-5} M) increased the content of cGMP of porcine coronary veins by about five- to six-fold. The effect of the nitrovasodilators was abolished by ODQ (Figure 4).

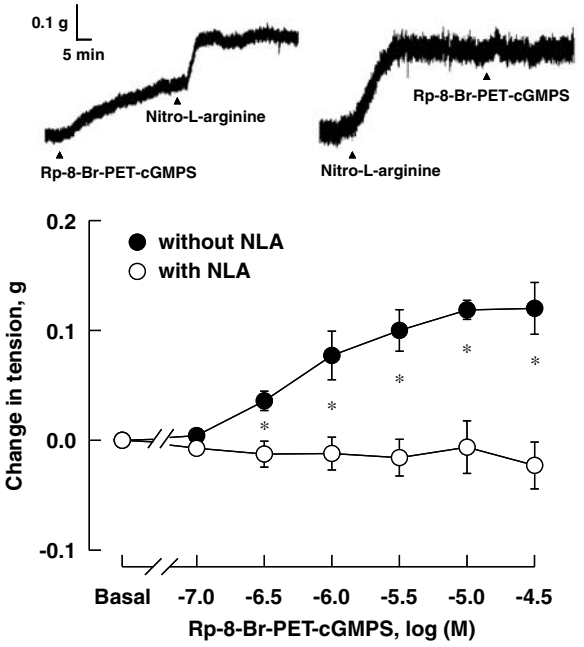

Figure 1 Upper panel: original traces of the effects of Rp-8-Br-PET-cGMPS (3×10^{-5} M) and nitro-L-arginine (10^{-4} M) on the basal tension of porcine coronary veins. Lower panel: concentration-dependent response of porcine coronary veins to Rp-8-Br-PET-cGMPS in the presence or absence of nitro-L-arginine (NLA, 10^{-4} M). Data are shown as means±s.e. mean; $n=4$–9 for each group. *Significantly different from vessels treated with NLA ($P<0.05$). Rp-8-Br-PET-cGMPS, β-phenyl-1, N^2-etheno-8-bromoguanosine-3′5′-cyclic monophosphorothioate, Rp-isomer.

PKG and PKA activity

The basal activities of PKG and PKA of porcine coronary veins with endothelium are shown in Figure 5. The basal activity of PKG was significantly inhibited by nitro-L-arginine (10^{-4} M), ODQ (3×10^{-5} M) and Rp-8-Br-PET-cGMPS (3×10^{-5} M), but was not affected by myristoylated PKI (6×10^{-6} M). The basal PKA activity was not affected by nitro-L-arginine (10^{-4} M), ODQ (3×10^{-5} M) and Rp-8-Br-PET-cGMPS (3×10^{-5} M), but was significantly inhibited by myristoylated PKI (6×10^{-6} M) (Figure 5).

The basal activity of PKG and PKA of porcine coronary veins without endothelium is shown in Figure 6. Nitroglycerin (3×10^{-5} M) and DETA NONOate (3×10^{-5} M) caused a

Table 1 Effects of pharmacological agents on the basal tension of porcine coronary veins

Treatments	*Without nitro-L-arginine*	*With nitro-L-arginine*
Control	ND	0.31±0.05
ODQ	0.30±0.03	0.32±0.04
Rp-8-Br-PET-cGMPS	0.12±0.02	0.28±0.05*
Indomethacin	0.09±0.05	0.39±0.06*
Myristoylated PKI	0.05±0.02	0.32±0.06*

ND, not determined; ODQ, 1H-[1,24]oxadiazolo[4,3-a]quinoxalin-1-one; Rp-8-Br-PET-cGMPS, β-phenyl-1, N^2-etheno-8-bromoguanosine-3′5′-cyclic monophosphorothioate, Rp-isomer.
The values shown are the mean (±s.e. mean) tension (in grams) from 7 to 14 samples for each condition. The concentrations used were: nitro-L-arginine, 10^{-4} M; ODQ, 3×10^{-5} M; Rp-8-Br-PET-cGMPS, 3×10^{-5} M; indomethacin, 10^{-5} M; myristoylated PKI, 6×10^{-6} M.
*Significant difference between vessels with and without nitro-L-arginine ($P<0.05$).

Table 2 Tension levels of porcine coronary veins raised by U46619 prior to determination of vasodilating effects of nitroglycerin, DETA NONOate, 8-Br-cGMP and 8-Br-cAMP

Vessel groups	*Increase in tension (g)*			*Myristoylated PKI*
	Control	*ODQ*	*Rp-8-Br-PET-cGMPS*	
Nitroglycerin study	3.20±0.94	2.59±0.40	2.54±0.24	3.14±0.31
DETA NONOate study	2.92±0.30	2.52±0.25	3.73±0.44	3.12±0.32
8-Br-cGMP study	2.68±0.46	ND	3.50±0.37	3.42±0.67
8-Br-cAMP study	2.67±0.42	ND	3.02±0.53	3.33±0.55

8-Br-cAMP, 8-bromo-adenosine 3′,5′-cyclic monophosphate; 8-Br-cGMP, 8-bromo-guanosine 3′,5′-cyclic monophosphate; ND, not determined; ODQ, 1H-[1,24]oxadiazolo[4,3-a]quinoxalin-1-one; Rp-8-Br-PET-cGMPS, β-phenyl-1, N^2-etheno-8-bromoguanosine-3′5′-cyclic monophosphorothioate, Rp-isomer.
The values shown are the mean (±s.e. mean) tension from 4 to 13 samples for each condition. All vessels were treated with nitro-L-arginine (10^{-4} M) and indomethacin (10^{-5} M). Other treatments were with ODQ, 3×10^{-5} M; Rp-8-Br-PET-cGMPS, 3×10^{-5} M; myristoylated PKI, 6×10^{-6} M. U46619 (10^{-7}–2×10^{-7} M) was used to increase the vessel tension to a constant level, about 60% of the maximal contraction obtained with U46619 (10^{-6} M; increase in tension: 5.17±0.21 g, $n=6$, $P<0.05$).
All values are expressed as means±s.e. mean, $n=4$–13.

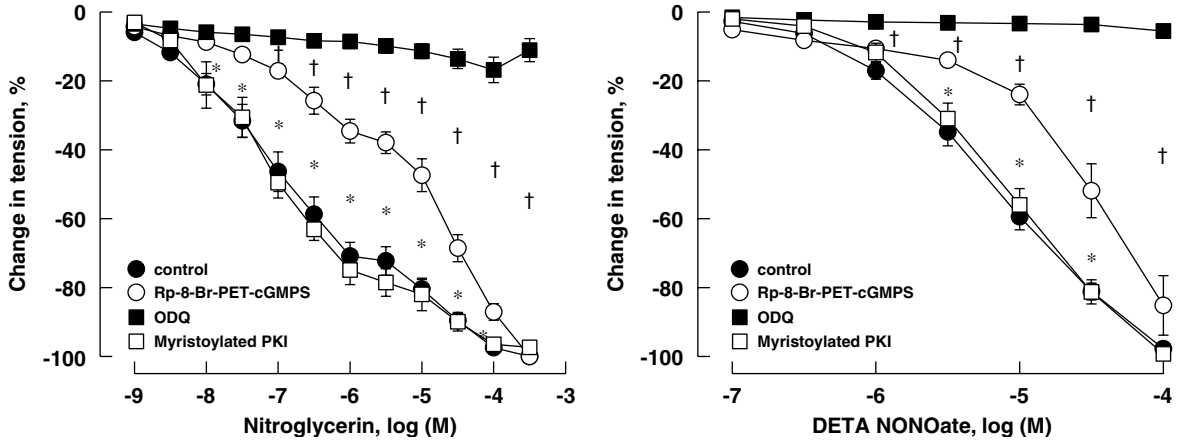

Figure 2 Relaxation of porcine coronary veins to nitroglycerin and DETA NONOate. Vessels were preconstricted to the same tension with U46619 (Table 2). Data are shown as means ± s.e. mean; $n = 4$–10 for each group. *Significant difference between control and those treated with Rp-8-Br-PET-cGMPS (3×10^{-5} M); †significant difference between control and those treated with ODQ (3×10^{-5} M) ($P < 0.05$). Rp-8-Br-PET-cGMPS, β-phenyl-1, N^2-etheno-8-bromoguanosine-3′5′-cyclic monophosphorothioate, Rp-isomer; ODQ, 1H-[1,24]oxadiazolo[4,3-a] quinoxalin-1-one.

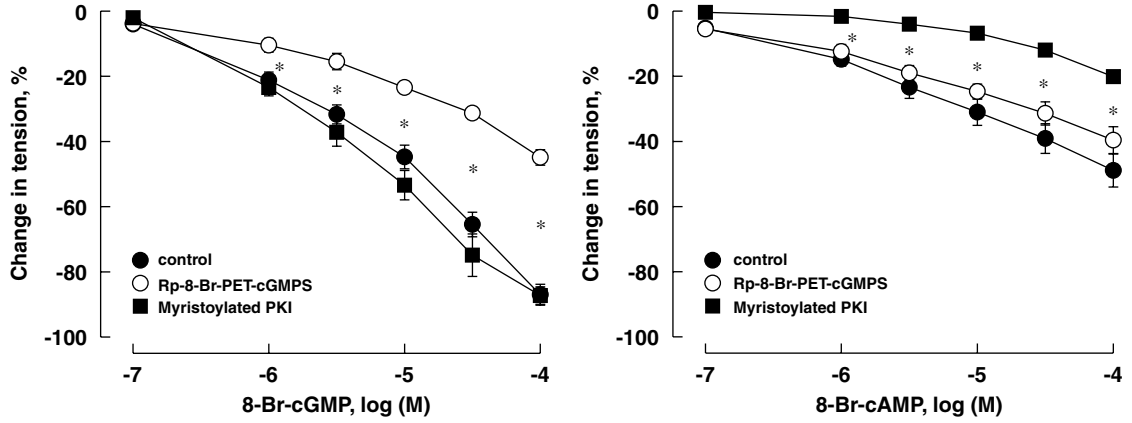

Figure 3 Relaxation of porcine coronary veins to 8-Br-cGMP and 8-Br-cAMP. Vessels were preconstricted with U46619 (Table 2). Data are shown as means ± s.e. mean; $n = 4$–13 for each group. *Significant difference between control and treated with Rp-8-Br-PET-cGMPS (3×10^{-5} M) (left panel) and treated with myristoylated PKI (6×10^{-6} M) (right panel) ($P < 0.05$). 8-Br-cAMP, 8-bromo-adenosine 3′,5′-cyclic monophosphate; 8-Br-cGMP, 8-bromo-guanosine 3′,5′-cyclic monophosphate; Rp-8-Br-PET-cGMPS, β-phenyl-1, N^2-etheno-8-bromoguanosine-3′5′-cyclic monophosphorothioate, Rp-isomer.

significant increase in the activity of PKG in these tissues and this effect was inhibited by Rp-8-Br-PET-cGMPS (3×10^{-5} M). The activity of PKA was not affected by nitroglycerin, DETA NONOate or Rp-8-Br-PET-cGMPS (Figure 6).

In the homogenates prepared from coronary veins, the increase in PKG activity caused by cGMP had a linear relationship up to 12 μg protein content (Figure 7). We therefore used 10 μg protein in our assays for PKG activity. PKG activity was stimulated by cGMP and cAMP in a concentration-dependent manner. Cyclic AMP was much less potent than cGMP as a stimulator for PKG, whereas cGMP was a much weaker stimulator for PKA (Figure 7).

The maximal increase in the activity of PKG of porcine coronary veins caused by cGMP (3.16 μM) was abolished by Rp-8-Br-PET-cGMPS (3×10^{-5} M) but was not affected by myristoylated PKI (6×10^{-6} M; Figure 8). This Figure also shows that the maximal increase in the activity of PKA of the veins caused by cAMP (3.16 μM) was largely abolished by myristoylated PKI (6×10^{-6} M) but was not affected by Rp-8-Br-PET-cGMPS (3×10^{-5} M).

Discussion

In porcine coronary veins, there is a continuous release of EDNO (Zhang *et al*., 2004). Consistent with this observation, we found that nitro-L-arginine, an inhibitor of nitric oxide synthase (Mülsch and Busse, 1990), caused a significant increase in the basal tension of porcine coronary veins, indicating that EDNO exerts a relaxing effect counteracting the basal constrictor activity. The effect of nitro-L-arginine could be fully prevented by ODQ, an inhibitor of soluble guanylyl cyclase (Garthwaite *et al*., 1995), suggesting that the inhibitory action exerted by EDNO on basal tension is

mediated primarily by cGMP. This postulation is further supported by a similar increase in basal tension of the veins induced by ODQ and the fact that the difference in basal cGMP content between coronary veins with and without endothelium was eliminated not only by nitro-L-arginine but also by ODQ. Some studies have shown that nitric oxide may exert its effect through cGMP-independent mechanisms such as nitrosylation of the target protein (Abderrahmane *et al*., 1998; Janssen *et al*., 2000). However, in the coronary veins, nitric oxide appears to work solely via the cGMP pathway. In our preliminary studies (H Qi and Y Gao, unpublished observation), contraction of coronary veins caused by nitro-L-arginine was not affected by the reducing agent dithiothreitol, which is widely used to investigate *S*-nitrosylation of various proteins (Väänänen *et al*., 2005; Han *et al*., 2006).

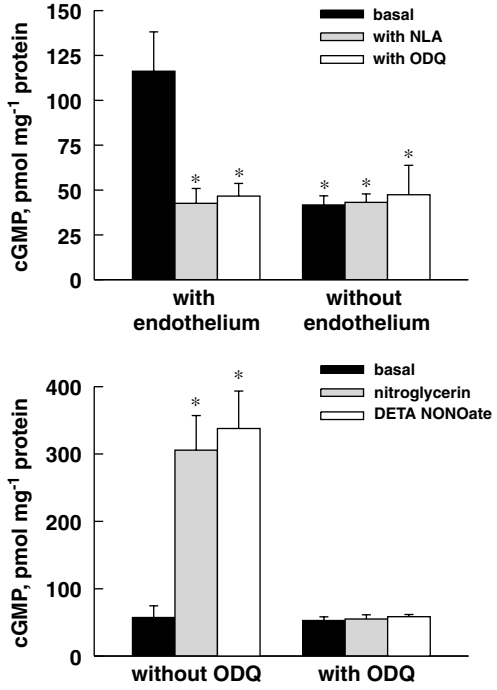

Figure 4 Upper panel: the intracellular content of cGMP of porcine coronary veins with and without endothelium under basal conditions, treated with nitro-L-arginine (NLA, 10^{-4} M) or ODQ (3×10^{-5} M). Data are shown as means±s.e. mean; $n=4$–9 for each group. *Significantly different from the basal value of vessels with endothelium ($P<0.05$). Lower panel: the intracellular content of cGMP of porcine coronary veins without endothelium stimulated with nitroglycerin (3×10^{-5} M) or DETA NONOate (3×10^{-5} M) in the absence or presence of ODQ (3×10^{-5} M). Data are shown as means±s.e. mean; $n=6$–10 for each group. *Significantly different from the basal value ($P<0.05$). ODQ, 1H-[1,24]oxadiazolo[4,3-a] quinoxalin-1-one.

Cyclic GMP may act through PKG-dependent and -independent mechanisms (Francis *et al*., 2005). In this study, the basal activity of PKG in coronary veins with endothelium was reduced by a similar extent after treatment with nitro-L-arginine or ODQ and also by Rp-8-Br-PET-cGMPS, a specific inhibitor of PKG (Butt *et al*., 1995). Hence, basal PKG activity in the veins that is activated by EDNO seems mainly to be due to activation of soluble guanylyl cyclase. However, the contraction induced by complete inhibition of PKG with Rp-8-Br-PET-cGMPS (3×10^{-5} M) was only about 38% of that induced by nitro-L-arginine. Thus, PKG activity may be only partially responsible for the effect of EDNO on the basal tension of veins. Another possible mode of action for cyclic GMP is by cross-activating PKA (Francis *et al*., 2005). Such a possibility, however, seems unlikely in our experiments as contraction of the coronary veins caused by nitro-L-arginine was not affected by myristoylated PKI, a specific inhibitor of PKA (Glass *et al*., 1989). Cyclic GMP may also affect smooth muscle tone by directly acting on ion channels such as potassium channels (Galindo *et al*., 2000, 2007). We found that $BaCl_2$ (3×10^{-5} M), 4-aminopyridine (3×10^{-3} M), charybdotoxin (10^{-7} M) and glibenclamide (10^{-5} M) (blockers of the delayed rectifier potassium channel, voltage-gated potassium channel, calcium-activated potassium channel, and ATP-sensitive potassium channel, respectively (Nelson and Quayle, 1995)) had no influence on contraction caused by nitro-L-arginine (H Qi and Y Gao, unpublished observation).Whether or not cGMP may cause its effect through other ion channels remains to be determined.

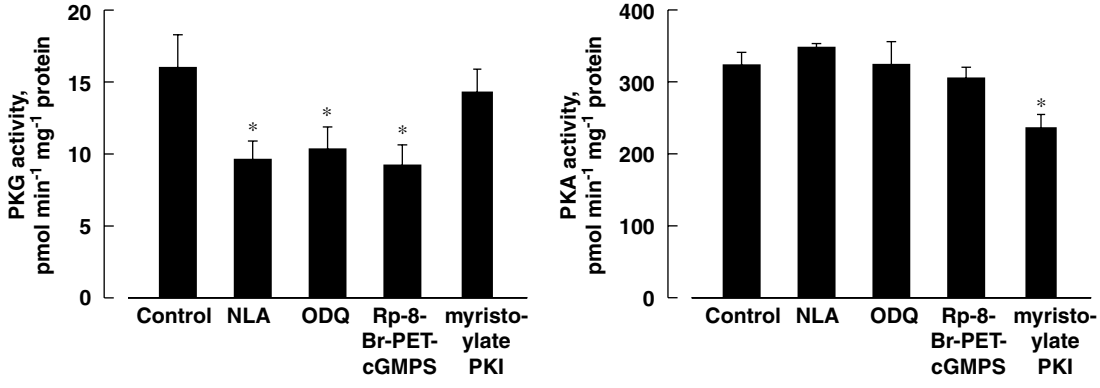

Figure 5 Effects of nitro-L-arginine (NLA, 10^{-4} M), ODQ (3×10^{-5} M), Rp-8-Br-PET-cGMPS (3×10^{-5} M) and myristoylated PKI (6×10^{-6} M) on the activity of PKG and PKA of porcine coronary veins with endothelium under basal conditions. Data are shown as means±s.e. mean; $n=10$–25 for each group. *Significantly different from control ($P<0.05$). Rp-8-Br-PET-cGMPS, β-phenyl-1, N^2-etheno-8-bromoguanosine-3′5′-cyclic monophosphorothioate, Rp-isomer; ODQ, 1H-[1,24]oxadiazolo[4,3-a]quinoxalin-1-one.

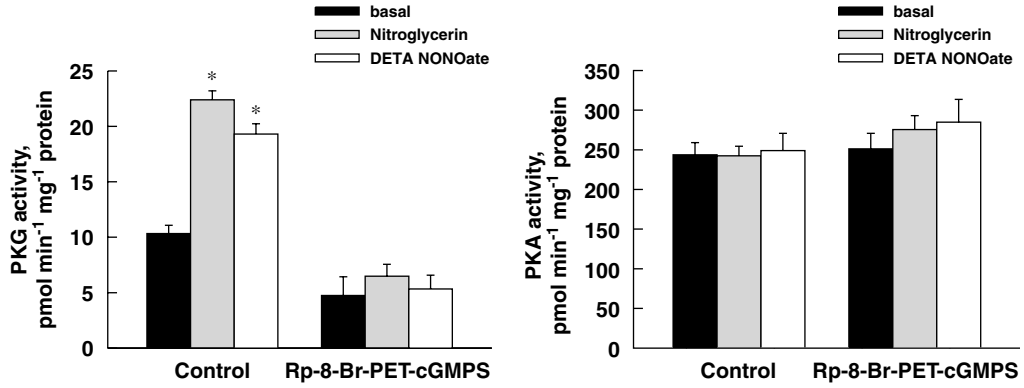

Figure 6 Effects of nitroglycerin (3×10^{-5} M) and DETA NONOate (3×10^{-5} M) on the activity of PKG and PKA of porcine coronary veins without endothelium in the absence or presence of Rp-8-Br-PET-cGMPS (3×10^{-5} M). Data are shown as means ± s.e. mean; $n = 7$–8 for each group. *Significantly different from basal ($P < 0.05$). Rp-8-Br-PET-cGMPS, β-phenyl-1, N^2-etheno-8-bromoguanosine-3′5′-cyclic monophosphorothioate.

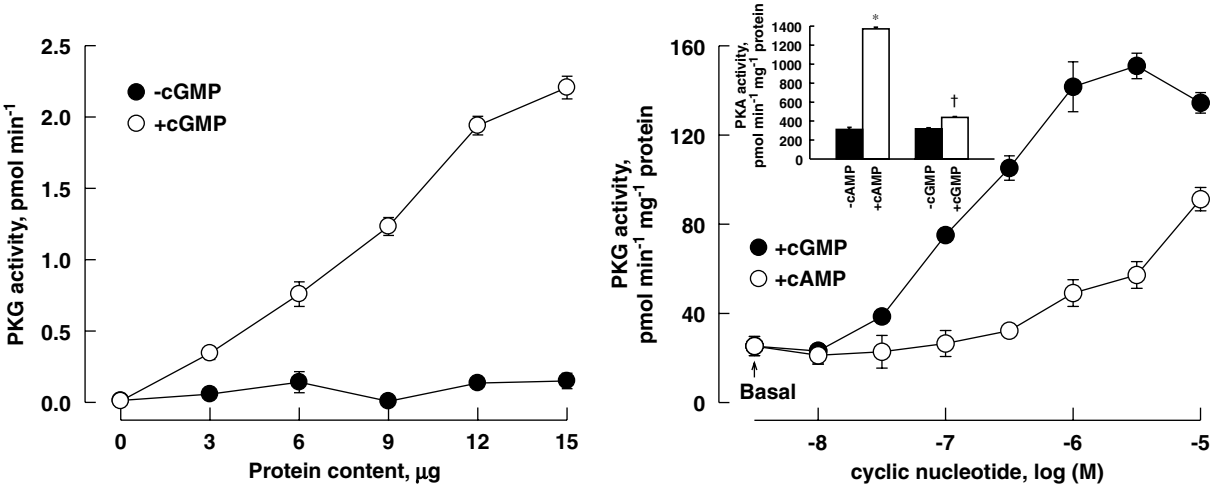

Figure 7 Left panel: PKG activity of the homogenates of porcine coronary veins using different concentrations of protein. Right panel: the concentration-dependent effects of cGMP and cAMP on PKG activity of the homogenates of porcine coronary veins. The inset shows the effects of cAMP and cGMP on PKA activity. −cGMP and −cAMP, without cGMP and cAMP, respectively; +cGMP and +cAMP, in the presence of cGMP and cAMP at 3.16 μM, respectively. Data are shown as means ± s.e. mean; $n = 4$ for each group.

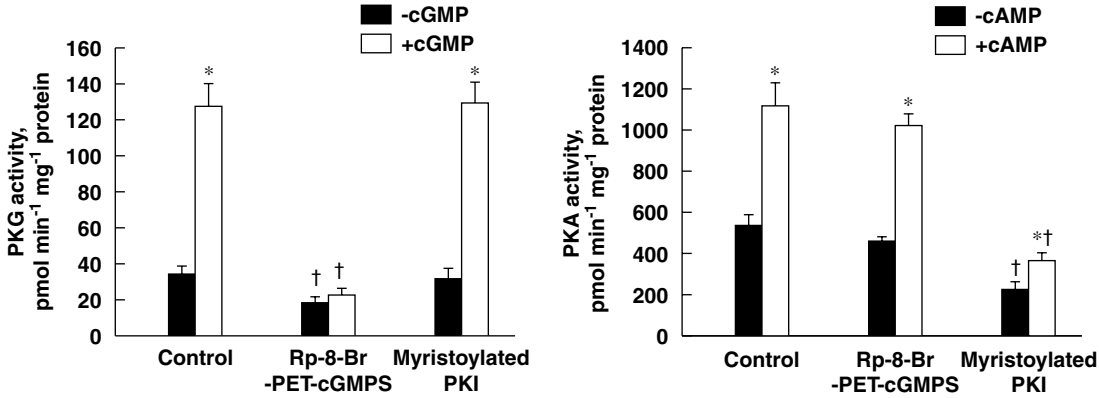

Figure 8 Effects of Rp-8-Br-PET-cGMPS (3×10^{-5} M) and myristoylated PKI (6×10^{-6} M) on PKG and PKA activity of the homogenate preparations of porcine coronary veins. −cGMP and −cAMP, without cGMP and cAMP, respectively; +cGMP and +cAMP, in the presence of cGMP and cAMP at 3.16 μM, respectively. Data are shown as means ± s.e. mean; $n = 4$ for each group. *Significantly different from activity without cGMP or cAMP ($P < 0.05$). †Significantly different from control ($P < 0.05$). Rp-8-Br-PET-cGMPS, β-phenyl-1, N^2-etheno-8-bromoguanosine-3′5′-cyclic monophosphorothioate.

In various vessel types, relaxation induced by endogenous and exogenous nitric oxide is mainly mediated by an increase in intracellular cGMP content resulting from activation of soluble guanylyl cyclase. The elevation in cGMP levels may activate PKG and thus cause vasodilation (Ignarro, 1990; Moncada *et al*., 1991). In this study, both the relaxation and elevation in cGMP levels in coronary veins induced by two nitric oxide donors, nitroglycerin and DETA NONOate, were largely eliminated by ODQ, indicating that the actions of these dilators were primarily mediated by cGMP via activation of soluble guanylyl cyclase. Relaxation of the veins caused by nitroglycerin, DETA NONOate and 8-Br-cGMP at EC_{50} was attenuated by Rp-8-Br-PET-cGMPS by 63, 66 and 51%, respectively. Hence, although cGMP may affect vascular tone by acting on phosphodiesterases and on cyclic nucleotide-gated ion channels (Lincoln *et al*., 2001; Hofmann *et al*., 2006), it appears that PKG is the primary mediator in nitrovasodilator-induced responses of porcine coronary veins. In this study, the effectiveness and specificity of Rp-8-Br-PET-cGMPS as an inhibitor of PKG was confirmed by the observation that activation of PKG in intact veins by nitroglycerin and DETA NONOate and in venous homogenates by cGMP, was fully inhibited at a concentration that did not affect PKA activity. We have found that the protein content of porcine coronary veins is markedly less than that of coronary arteries (Qin *et al*., 2007). However, the responses of veins to nitroglycerin, DETA NONOate and 8-Br-cGMP were comparable to those of coronary arteries. Therefore, the absolute PKG concentration may not predict the importance of PKG in a tissue because PKG at low concentrations may still catalyse critical phosphorylation events (Francis and Corbin, 1994).

Although cGMP may exert its effect through activation of PKG, it may also activate PKA (Francis *et al*., 1988; Lincoln *et al*., 1990). For instance, relaxation of guinea-pig tracheal smooth muscle induced by 8-Br-cGMP is partially blocked by inhibitors of PKA (Algara-Suarez and Espinosa-Tanguma, 2004). In our present study, PKA activity in venous homogenate was moderately stimulated by cGMP in comparison to its stimulation by cAMP. However, the PKA activity in isolated coronary veins was not significantly affected by either nitroglycerin or DETA NONOate at concentrations that had induced about 85% relaxation. Moreover, myristoylated PKI, a specific inhibitor of PKA (Glass *et al*., 1989), at a concentration that reduces PKA activity below basal levels, did not significantly affect either basal tension or the relaxations induced by nitroglycerin, DETA NONOate or 8-Br-cGMP. These results suggest that, at least under physiological conditions, PKA does not appear to be critically involved in the regulation of basal tension in porcine coronary veins nor be involved in relaxation induced by nitrovasodilators and cGMP. These results are consistent with results from our previous studies as well as of others (Gao *et al*., 1999; Dhanakoti *et al*., 2000; Bonnevier *et al*., 2004; Qin *et al*., 2007). In PKG I knockout mice but not in normal mice, relaxation of aorta induced by DEA-NO was affected by PKA inhibitor Rp-8-Br-cAMPS (Sausbier *et al*., 2000). Studies show that cGMP cross-activates PKA at about a 1000-fold higher concentration (Kawada *et al*., 1997; Lee *et al*., 1997; Bonnevier *et al*., 2004) and it is postulated that cGMP-dependent activation of PKA may not be operative under physiological conditions. Under pathophysiological circumstances when nitric oxide production is very high, activation of PKA by cGMP may act as an additional regulatory mechanism that produces generalized hypotension (Sausbier *et al*., 2000; Bonnevier *et al*., 2004).

The coronary venous system has largely been considered as a passive conduit for coronary blood to flow into the right atrium. However, as coronary veins are connected with coronary arteries and capillaries in series and as coronary veins exhibit marked responses to various vasoactive agents, the veins may actively contribute to the regulation of the coronary circulation (Chilian *at al*., 1989; Cocks *et al*., 1989a, b; Gulbenkian *et al*., 1994; Banitt *et al*., 1995; Saetrum Opgaard *et al*., 1995, 1996; Saetrum Opgaard and Edvinsson, 1997; Zhang *et al*., 2004). Studies show that coronary veins may be more sensitive to noradrenaline, ATP and endothelin-1 than coronary arteries (Cocks *et al*., 1989a, b; Saetrum Opgaard and Edvinsson, 1997). Hence, under some circumstances, coronary veins may play a greater role than the arteries in regulating coronary blood flow. With regard to the role of EDNO in the coronary venous system, there is a study showing that flow-induced dilation of porcine coronary venules is mediated in an EDNO-dependent manner and that the venular dilation induced by flow is converted to constriction when the endothelium is denuded (Kuo *et al*., 1993). This suggests that EDNO may play an important role in ensuring adequate blood flow and oxygen transport to the heart during intense metabolic demands (Kuo *et al*., 1993). In this study, nitroglycerin and nitric oxide caused a marked relaxation of porcine coronary veins, which is comparable to that of coronary arteries (Qin *et al*., 2007). Our study suggests that the relaxation of porcine coronary veins to nitrovasodilators is primarily mediated by PKG. If applicable to humans, it may be of clinical significance since augmentation of PKG may be a therapeutic target to relax the coronary veins and improve coronary blood flow.

Acknowledgements

This study was supported in part by the National Natural Science Foundation of China, Grant no. 30370523, no. 30470629, the Major National Basic Research Program of P. R. China (no. 2006CB503802), and the National Institute of Health Grants HL-059435 and HL-075187, USA.

Conflict of interest

The authors state no conflict of interest.

References

Abderrahmane A, Salvail D, Dumoulin M, Garon J, Cadieux A, Rousseau E (1998). Direct activation of K_{Ca} channel in airway smooth muscle by nitric oxide: involvement of a nitrothiosylation mechanism? *Am J Respir Cell Mol Biol* **19**: 485–497.

Algara-Suarez P, Espinosa-Tanguma R (2004). 8-Br-cGMP mediates relaxation of tracheal smooth muscle through PKA. *Biochem Biophys Res Commun* **314**: 597–601.

Banitt PF, Dai HB, Wang SY, Friedman M, Sellke FW (1995). Myogenic and agonist induced responses of coronary venules after cold hyperkalaemic cardioplegia. *Cardiovasc Res* **29**: 827–833.

Bonnevier J, Fässler R, Somlyo AP, Somlyo AV, Arner A (2004). Modulation of Ca^{2+} sensitivity by cyclic nucleotides in smooth muscle from protein kinase G-deficient mice. *J Biol Chem* **279**: 5146–5151.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal Chem* **72**: 249–254.

Butt E, Pohler D, Genieser HG, Huggins JP, Bucher B (1995). Inhibition of cyclic GMP-dependent protein kinase-mediated effects by (Rp)-8-bromo-PET-cyclic GMPS. *Br J Pharmacol* **116**: 3110–3116.

Cassin S (1980). Role of prostaglandins and thromboxanes in the control of the pulmonary circulation in the fetus and newborn. *Semin Perinatol* **4**: 101–107.

Chilian WM, Layne SM, Eastham CL, Marcus ML (1989). Heterogeneous microvascular coronary alpha-adrenergic vasoconstriction. *Circ Res* **64**: 376–388.

Cocks TM, Broughton A, Dib M, Sudhir K, Angus JA (1989a). Endothelin is blood vessel selective: studies on a variety of human and dog vessels *in vitro* and on regional blood flow in the conscious rabbit. *Clin Exp Pharmacol Physiol* **16**: 243–246.

Cocks TM, Faulkner NL, Sudhir K, Angus J (1989b). Reactivity of endothelin-1 on human and canine large veins compared with large arteries *in vitro*. *Eur J Pharmacol* **171**: 17–24.

Colbran JL, Francis SH, Leach AB, Thomas MK, Jiang H, McAllister LM *et al*. (1992). A phenylalanine in peptide substrates provides for selectivity between cGMP- and cAMP-dependent protein kinases. *J Biol Chem* **267**: 9589–9594.

Coleman RA, Humphrey PP, Kennedy I, Levy GP, Lumley P (1981). Comparison of the actions of U-46619, a prostaglandin H2-analogue, with those of prostaglandin H2 and thromboxane A2 on some isolated smooth muscle preparations. *Br J Pharmacol* **73**: 773–778.

Dhanakoti S, Gao Y, Nguyen MQ, Raj JU (2000). Involvement of cGMP-dependent protein kinase in the relaxation of ovine pulmonary arteries to cGMP and cAMP. *J Appl Physiol* **88**: 1637–1642.

Drexler H (1999). Nitric oxide and coronary endothelial dysfunction in humans. *Cardiovasc Res* **43**: 572–579.

Francis SH, Blount MA, Zoraghi R, Corbin JD (2005). Molecular properties of mammalian proteins that interact with cGMP: protein kinases, cation channels, phosphodiesterases, and multidrug anion transporters. *Front Biosci* **10**: 2097–2117.

Francis SH, Corbin JD (1994). Progress in understanding the mechanism and function of cyclic GMP-dependent protein kinase. *Adv Pharmacol* **26**: 115–170.

Francis SH, Noblett BD, Todd BW, Wells JN, Corbin JD (1988). Relaxation of vascular and tracheal smooth muscle by cyclic nucleotide analogs that preferentially activate purified cGMP-dependent protein kinase. *Mol Pharmacol* **34**: 506–517.

Galindo BE, Beltrán C, Cragoe EJ, Darszon A (2000). Participation of a K(+) channel modulated directly by cGMP in the speract-induced signaling cascade of strongylocentrotus purpuratus sea urchin sperm. *Dev Biol* **221**: 285–294.

Galindo BE, de la Vega-Beltran JL, Labarca P, Vacquier VD, Darszon A (2007). Sp-tetraKCNG: A novel cyclic nucleotide gated K(+) channel. *Biochem Biophys Res Commun* **354**: 668–675.

Gao Y, Dhanakoti S, Tolsa J-F, Raj JU (1999). Role of protein kinase G in nitric oxide- and cGMP-induced relaxation of newborn ovine pulmonary veins. *J Appl Physiol* **87**: 993–998.

Gao Y, Zhou H, Ibe BO, Raj JU (1996). Prostaglandins E_2 and I_2 cause greater relaxations in pulmonary veins than in arteries of newborn lambs. *J Appl Physio* **81**: 2534–2539.

Gao Y, Zhou H, Raj JU (1995). Endothelium-derived nitric oxide plays a larger role in pulmonary veins than in arteries of newborn lambs. *Cir Res* **76**: 559–565.

Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K, Mayer B (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol Pharmacol* **48**: 184–188.

Glass DB, Cheng HC, Mende-Mueller L, Reed J, Walsh DA (1989). Primary structural determinants essential for potent inhibition of cAMP-dependent protein kinase by inhibitory peptides corresponding to the active portion of the heat-stable inhibitor protein. *J Biol Chem* **264**: 8802–8810.

Gordon AM, Huxley AF, Julian FJ (1966). The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *J Physiol (London)* **184**: 170–192.

Gulbenkian S, Saetrum Opgaard O, Barroso CP, Wharton J, Polak JM, Edvinsson L (1994). The innervation of guinea pig epicardial coronary veins: Immunohistochemistry, ultrastructure and vasomotility. *J Auton Nerv Syst* **47**: 201–212.

Han NL, Ye JS, Yu AC, Sheu FS (2006). Differential mechanisms underlying the modulation of delayed-rectifier K^+ channel in mouse neocortical neurons by nitric oxide. *J Neurophysiol* **95**: 2167–2178.

Hofmann F, Feil R, Kleppisch T, Schlossmann J (2006). Function of cGMP-dependent protein kinases as revealed by gene deletion. *Physiol Rev* **86**: 1–23.

Ignarro LJ (1990). Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annu Rev Pharmacol Toxicol* **30**: 535–560.

Janssen LJ, Premji M, Lu-Chao H, Cox G, Keshavjee S (2000). NO(+) but not NO radical relaxes airway smooth muscle via cGMP-independent release of internal Ca(2+). *Am J Physiol* **278**: L899–L905.

Kawada T, Toyosato A, Islam MO, Yoshida Y, Imai S (1997). cGMP-kinase mediates cGMP- and cAMP-induced Ca2+ desensitization of skinned rat artery. *Eur J Pharmacol* **323**: 75–82.

Kuo L, Arko F, Chilian WM, Davis MJ (1993). Coronary venular responses to flow and pressure. *Circ Res* **72**: 607–615.

Lee MR, Li L, Kitazawa T (1997). Cyclic GMP causes Ca^{2+} desensitization in vascular smooth muscle by activating the myosin light chain phosphatase. *J Biol Chem* **272**: 5063–5068.

Lincoln TM, Cornwell TL, Taylor AE (1990). cGMP-dependent protein kinase mediates the reduction of Ca^{2+} by Camp in vascular smooth muscle cells. *Am J Physiol* **258**: C399–C407.

Lincoln TM, Dey N, Sellak H (2001). Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J Appl Physiol* **91**: 1421–1430.

Lohmann SM, Vaandrager AB, Smolenski A, Walter U, De Jonge HR (1997). Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem Sci* **22**: 307–312.

Meyer Jr RB, Miller JP (1974). Analogs of cyclic AMP and cyclic GMP: general methods of synthesis and the relationship of structure to enzymic activity. *Life Sci* **14**: 1019–1040.

Moncada S, Palmer RM, Higgs EA (1991). Nitric oxide: physiology, pathology, and pharmacology. *Pharmacol Rev* **43**: 109–142.

Mülsch A, Busse R (1990). N^G-nitro-L-arginine (N^S-[imino (nitroamino) methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmiedeberg's Arch Pharmacol* **341**: 143–147.

Nelson MT, Quayle JM (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol* **268**: C799–C822.

Qin X, Zheng X, Qi H, Dou D, Raj JU, Gao Y (2007). cGMP-dependent protein kinase in regulation of basal tone and in nitroglycerin- and nitric-oxide-induced relaxation in porcine coronary artery. *Pflugers Arch Eur J Physiol* **454**: 913–923.

Saetrum Opgaard O, Cantera L, Adner M, Edvinsson L (1996). Endothelin-A and -B receptors in human coronary arteries and veins. *Regul Pept* **63**: 149–156.

Saetrum Opgaard O, Edvinsson L (1997). Mechanical properties and effects of sympathetic co-transmitters on human coronary arteries and veins. *Basic Res Cardiol* **92**: 168–180.

Saetrum Opgaard O, Gulbenkian S, Bergdahl A, Barroso CP, Andrade NC, Polak JM *et al.* (1995). Innervation of human epicardial coronary veins: immunohistochemistry and vasomotility. *Cardiovasc Res* **29**: 463–468.

Sausbier M, Schubert R, Voigt V, Hirneiss C, Pfeifer A, Korth M *et al*. (2000). Mechanisms of NO/cGMP-dependent vasorelaxation. *Circ Res* **87**: 825–830.

Tiefenbacher CP, Chilian WM (1998). Heterogeneity of coronary vasomotion. *Basic Res Cardiol* **93**: 446–454.

Tune JD, Gorman MW, Feigl EO (2004). Matching coronary blood flow to myocardial oxygen consumption. *J Appl Physiol* **97**: 404–415.

Väänänen AJ, Kankuri E, Rauhala P (2005). Nitric oxide-related species-induced protein oxidation: Reversible, irreversible, and protective effects on enzyme function of papain. *Free Radic Biol Med* **38**: 1102–1111.

Walsh MP, Kargacin GJ, Kendrick-Jones J, Lincoln TM (1995). Intracellular mechanisms involved in the regulation of vascular smooth muscle tone. *Can J Physiol Pharmacol* **73**: 565–573.

Wang X, Tong M, Chinta S, Raj JU, Gao Y (2006). Hypoxia-induced reactive oxygen species downregulate ET_B-receptor mediated contraction of rat pulmonary arteries. *Am J Physiol* **290**: L570–L578.

Zhang RZ, Yang Q, Yim AP, Huang Y, He GW (2004). Different role of nitric oxide and endothelium-derived hyperpolarizing factor in endothelium-dependent hyperpolarization and relaxation in porcine coronary arterial and venous system. *J Cardiovasc Pharmacol* **43**: 839–850.