J Physiol Sci (2010) 60:389–397
DOI 10.1007/s12576-010-0107-x

ORIGINAL PAPER

Vasoactive intestinal polypeptide relaxes isolated rat pulmonary artery rings through two distinct mechanisms

Shuang Zhang · Yun Liu · Shouli Guo · Jianing Zhang · Xiaojie Chu · Chun Jiang · Daling Zhu

Received: 14 May 2010 / Accepted: 20 July 2010 / Published online: 7 August 2010

Abstract Vasoactive intestinal polypeptide (VIP), an endogenous neuropeptide normally present in lungs and other organs, relaxes pulmonary arteries (PAs) in different species, whereas the underlying mechanisms are still not fully understood. The aim of this study, therefore, is to investigate the signal transduction of VIP in the relaxation of isolated rat PA rings. The isometric tension of the rings was studied in vitro with force-electricity transducers. In endothelium-intact (EI) rings, VIP elicited concentration-dependent relaxation after the rings were pre-contracted by phenylephrine. A similar effect, though smaller, was observed in endothelium-denuded (ED) rings. Inhibition of the endothelial nitric oxide synthase (eNOS) by NG-nitro-L-arginine methyl ester diminished the VIP-induced vasodilatation of PA rings. The VIP-induced vasorelaxation was markedly reduced by the inhibition of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway with wortmannin and LY294002, respectively, which was seen in EI rings, but not in ED rings. Western blot analysis revealed that VIP increased the phosphorylation of eNOS at Ser 1177, but did not affect the overall expression of eNOS. In ED rings, the PKA inhibitor H-89 and K_{ATP} channel inhibitor glibenclamide almost totally abolished the vasodilatation effect of VIP. The results suggested that the vasodilatation effect of VIP on rat PAs is mediated by both vascular endothelium and smooth muscle, involving respectively the PI3K/Akt-eNOS pathway and the PKA-K_{ATP} channel pathway.

Keywords VIP · PA rings · PI3K/Akt · eNOS · PKA · K_{ATP} channel

Abbreviations

VIP	vasoactive intestinal polypeptide
PAs	pulmonary arteries
EI	endothelium-intact
PE	phenylephrine
ED	endothelium-denuded
eNOS	nitric oxide synthase
L-NAME	NG-nitro-L-arginine methyl ester
PI3K/Akt	phosphatidylinositol 3-kinase/protein kinase B
SM	smooth muscle
VSM	vascular smooth muscle
NO	nitric oxide
PPH	primary pulmonary hypertension
MA	mesenteric artery
AC	adenylyl cyclase
p-eNOS	phosphorylated eNOS
p-eNOS-Ser^{1177}	phosphorylated eNOS at Ser1177
PAEC	pulmonary arterial endothelial cell

S. Zhang · Y. Liu · S. Guo · J. Zhang · X. Chu · D. Zhu (✉)
Department of Biopharmaceutical Sciences,
College of Pharmacy, Harbin Medical University,
157 Baojian Road, Nangang District, Harbin 150081,
Heilongjiang, People's Republic of China
e-mail: dalingz@yahoo.com

D. Zhu
Biopharmaceutical Key Laboratory of Heilongjiang
Province, Harbin 150081, China

D. Zhu
Biopharmaceutical Key Laboratory of Ministry
of Education, Harbin, China

C. Jiang
Department of Biology, Georgia State University,
24 Peachtree Center Avenue, Atlanta, GA 30302-4010, USA

PBS	phosphate-buffered saline
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum

Introduction

Primary pulmonary hypertension (PPH) is a progressive and life-threatening disease. Timely diagnosis is necessary but is often not achieved, as the initial symptoms are mild, requiring subtle examinations. Its prognosis is rather poor, with an average survival rate of 2.8 years without therapeutic intervention [1, 2]. Impairment of endothelial homeostasis has been revealed, including a reduced synthesis of prostacyclin and nitric oxide (NO) [3, 4] and an increased production of thromboxane and endothelin-1 [3, 5]. Prostacyclin and its analogues are effective vasodilators. They are also inhibitors of both platelet aggregation and vascular SM (VSM) cell proliferation [6, 7]. Thus, they have been applied for the PPH treatment [8, 9]. However, most prostanoids are pharmacologically unstable, an aspect that restricts their application, especially when given continuously for a prolonged period. Therefore, people have been searching for alternative treatments of PPH, especially endogenous vasodilators that have more favorable pharmacokinetic and pharmacodynamic features. Among various endogenous vasodilators in the human body is the vasoactive intestinal polypeptide (VIP) whose pharmacological characteristics seem suitable to combat PPH. Indeed, clinical studies have shown that as a new concept in management of PPH, VIP results in substantial improvement of hemodynamic and prognostic parameters of the disease without evident side effects [10].

The VIP is one of the most abundant, biologically active peptides in human lungs. It is a likely neurotransmitter or neuromodulator of the non-adrenergic non-cholinergic nervous system in airways and affects many aspects of pulmonary biology [11]. The VIP induces vasodilatation of mesenteric arteries (MAs) via activation of the Gs-adenylyl cyclase (AC) PKA pathway in VSM [12]. Recent studies have demonstrated that the relaxation effect of VIP on isolated rat PAs depends in part on the presence of the endothelium involving endothelial NO [13] and/or cyclooxygenase metabolites [14]. However, the essential signaling pathways for the VIP-induced PAs vasodilatation are still unclear. To address this issue, we employed tension studies of PA rings to determine the effect of endothelium and VSM as well as the potential mechanism involved in the VIP-induced PAs vasodilatation. Our results suggest that the VIP-induced vasodilatation is mediated by both endothelium and VSM via distinct intracellular signaling systems.

Methods

Reagents

Vasoactive intestinal polypeptide was obtained from Biosynthesis Biotechnology Co. (Beijing, China): PE, L-NAME, wortmannin, LY294002, carbamylcholine chloride and H-89 were obtained from Sigma Chemical Co. (St Louis, MO); phosphorylated eNOS (p-eNOS) at Ser1177 (p-eNOS-Ser^{1177}) antibody and eNOS antibody were from Cell Signaling Technology, Inc. (Danvers, MA), and polyclonal actin antibody from Santa Cruz Biotechnology, Inc. (Sanata Cruz, CA). All other reagents were from common commercial sources.

Animals

Eleven male Wistar rats weighing 220–350 g were used in the studies that fully comply with regulations of the Institutional Animal Care and Use Committee of Harbin Medical University. The rats were housed in the Animal Research Center at a controlled ambient temperature of 22 ± 2°C with 50 ± 10% relative humidity and a 12-h light-dark cycle. After the rats were deeply anesthetized with an intraperitoneal injection of the 1% chloral hydrate according to their weight, the thoracic chest was opened, and the heart and lungs were removed en bloc and placed in the cold Krebs solution. The secondary PAs (1–1.5 mm in diameter) were then dissected free, cut into 3-mm rings and maintained in the cold Krebs solution (see below). During the procedure, attention was paid to avoid stretching the vessel unnecessarily and contacting the luminal surface to ensure the endothelial integrity.

Tension studies of PA rings

The experiments were carried out as detailed in our previous report [15]. Briefly, each PA ring was mounted on a force transducer (ALC-MPA, Shanghai Alcbio Biology Technology Co., Ltd., China) and placed in a water-jacketed organ bath. The system allows simultaneous recordings from eight rings. The rings were bathed with 2 ml oxygenated Krebs solution containing (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.57, KH_2PO_4 1.2, $NaHCO_3$ 24 and D-glucose 10 (pH 7.4, at 37°C). The 0.25–0.35 g preload was added to the rings followed by a 30–40-min equilibration. In the case of endothelium-denuded preparations, the vessels were mechanically rubbed by a wolfram wire before equilibration. Endothelial integrity was examined in all experiments by the presence of the characteristic relaxation response to carbamylcholine chloride (5×10^{-6} M). After equilibration, each ring was contracted with 10^{-6} M PE followed by treatment with carbamylcholine chloride. Vascular rings

displaying less than 10% relaxation to carbamylcholine chloride were considered as endothelium-denuded.

Cell culture

Neonatal bovine was used in the studies in full compliance with the Ethics Committee of Laboratory Animals at Harbin Medical University. Primary cultures of pulmonary arterial endothelial cells (PAECs) were prepared as previously described [16]. In brief, pulmonary conduct arteries were isolated from lungs of neonatal bovine obtained at a local abattoir, slit open along their lengths, and washed with phosphate-buffered saline (PBS) to remove blood. The vessels were dissected free of fat and excess adventitial tissue, and PAECs were scraped from PAs of neonatal bovine and cultured in 10-cm Petri dishes in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin, then cultured in a 37°C, 5% CO_2 humidified incubator for 3–5 days. Before each experiment, the cells were incubated in 0.3% FBS-DMEM for 12–24 h to stop cell growth. Cell viability (usually >98%) was determined by Trypan blue exclusion.

Experimental protocols

Protocol 1

The effect of VIP on rat EI and ED PA rings: To study vasorelaxation properties of VIP in the PA rings with and without endothelium, the rings were pre-contracted with PE (10^{-6} M). When the contraction reached a steady state, cumulative concentration-response curves to VIP were produced by adding increasing concentrations of VIP (10^{-9} to 10^{-5} M).

Protocol 2

The effect of eNOS on VIP-induced relaxation: To test the hypothesis that the VIP-induced vasodilatation is mediated through the eNOS signaling pathway, we pre-incubated the EI and ED PA rings with L-NAME (10^{-4} M) in the organ bath for 20 min before 10^{-6} M PE was injected directly into the bath, then the effects of VIP were examined.

Protocol 3

Influence of the PI3K/Akt pathway on PA relaxation induced by VIP: This set of experiments was undertaken to investigate the hypothesis that the VIP-induced vasodilatation is mediated by the PI3K/Akt signaling system. After EI and ED PA rings were incubated with wortmannin (10^{-7} M) or LY294002 (3×10^{-5} M) for 20 min respectively in an organ bath, 10^{-6} M PE was added directly into the bath to investigate the effects of VIP on PA rings.

Protocol 4

The levels of eNOS and phosphorylated eNOS were studied in cultured bovine PAECs with Western blotting. After treatment with VIP for 5–7 min, the cells were washed twice in ice-cold PBS. Proteins were solubilized and extracted with 300 μl lysis buffer (Tris 50 mM, pH 7.4, NaCl 150 mM, Triton X-100 1%, EDTA 1 mM, and PMSF 2 mM) and incubated at 4°C for 30 min. The lysates were sonicated and centrifuged at 14,000*g* for 15 min, and the insoluble fraction was discarded. The supernatants were collected and stored at −80°C. Protein concentrations were determined by the Bradford protein assay. Equal amounts (50 μg) of protein from each sample were mixed and boiled in SDS-PAGE sample buffer for 5 min. The protein was subjected to electrophoresis on 8% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore, USA) in a Mini Trans-Blot transfer apparatus (Bio-Rad) under conditions recommended by the manufacturer. After incubation overnight at 4°C in a blocking buffer (0.1% Tween 20 in PBS) containing 5% nonfat dry milk powder, the membranes were incubated with commercially available polyclonal antibodies specific for p-eNOS-Ser^{1177} and eNOS, respectively. The concentrations of polyclonal p-eNOS-Ser^{1177} (1:1000 dilution) and eNOS (1:500 dilution) antibodies were used according to the manufacturer's instructions. Blots were then washed and incubated with anti-rabbit horseradish peroxidase-conjugated IgG for 120 min at room temperature. The bound antibody was detected with an enhanced chemiluminescence detection system (Amersham). To adjust for loading differences, blots were reprobed with a monoclonal antibody to β-actin (Sigma).

Protocol 5

The effect of PKA and K_{ATP} channels on VIP-induced relaxation of VSM: To reveal the mechanism of VIP-induced relaxation of PA VSM, we incubated ED rings with H-89 (5×10^{-7} M), the selective and potent inhibitor of PKA (Sigma), for 30 min and glibenclamide (10^{-5} M) for 20 min, respectively. Then 10^{-6} M PE was added directly into the organ bath, followed by 10^{-6} M VIP.

Effects of several agents on the VIP-produced vasorelaxation were analyzed by comparison of the vasorelaxation after agent exposure with control produced by 10^{-6} M VIP when the maximum vasodilatation was elicited in EI and ED rings.

Statistical analysis

The magnitude of vasorelaxation was expressed in g (mean ± SE), and n is the number of rings. The relaxation was expressed as the percentage decrease in vascular tones from the plateau of pre-contraction with PE (10^{-6} M). Mean values among the experimental groups were examined using two-tailed analyses of variance (ANOVA) followed by Dunnetts's or Student's *t* tests. The differences were considered statistically significant when $P \leq 0.05$.

Results

Two distinct mechanisms for the PA relaxation induced by VIP

After 30–40 min equilibration with a 0.25–0.35-g preload, the PA rings were exposed to 10^{-6} M PE. The exposure produced rapid contraction of these rings by 0.4–0.6 g. The peak contraction was reached in 4–5 min and maintained for at least 20 min without evident decline. During the vasoconstriction, administration of VIP (10^{-9} to 10^{-5} M) produced concentration-dependent relaxations of EI rings ($n = 6$, Fig. 1a, b). The relationship of force versus VIP concentration showed a clear sigmoid curve that can be described with the Hill equation showing the IC_{50} 10^{-8} M and the Hill coefficient (*h*) 1.0 (Fig. 1c). At the maximum effect, VIP (10^{-6} M) relaxed the pre-contracted EI rings by 46.7 ± 6.8%.

After removal of the endothelium, VIP remained, relaxing the PA rings, although its vasodilatation effect was significantly reduced. The maximum vasodilatation in EI and ED was produced by 10^{-6} M VIP. Thus, this concentration of VIP (10^{-6} M) was used in the following PA ring studies. Interestingly, not only was the maximum effect decreased to 22.1 ± 2.8% ($n = 6$, Fig. 1a, b), but also the IC_{50} was raised to 9×10^{-8} M and $h = 1.2$ (Fig. 1d). These results thus suggest that VIP is an effective PA relaxant, and its action involves not only the VSM, but also the PA endothelium.

The endothelium effect relied on the PI3K-Akt-eNOS system

Effect of eNOS

NO is a major player in the endothelium-dependent vasodilatation, although vascular endothelium also releases other vasorelaxants. Therefore, we studied the NO system in the VIP-induced PA ring's relaxation. In EI rings, PE produced the same degree of vasoconstriction after a preincubation with the eNOS inhibitor L-NAME (10^{-4} M) for 20 min. After the peak tension was reached, 10^{-6} M VIP only reduced the vascular tension by 21.5 ± 3.5% ($n = 6$), a level that was about 50% lower than without L-NAME

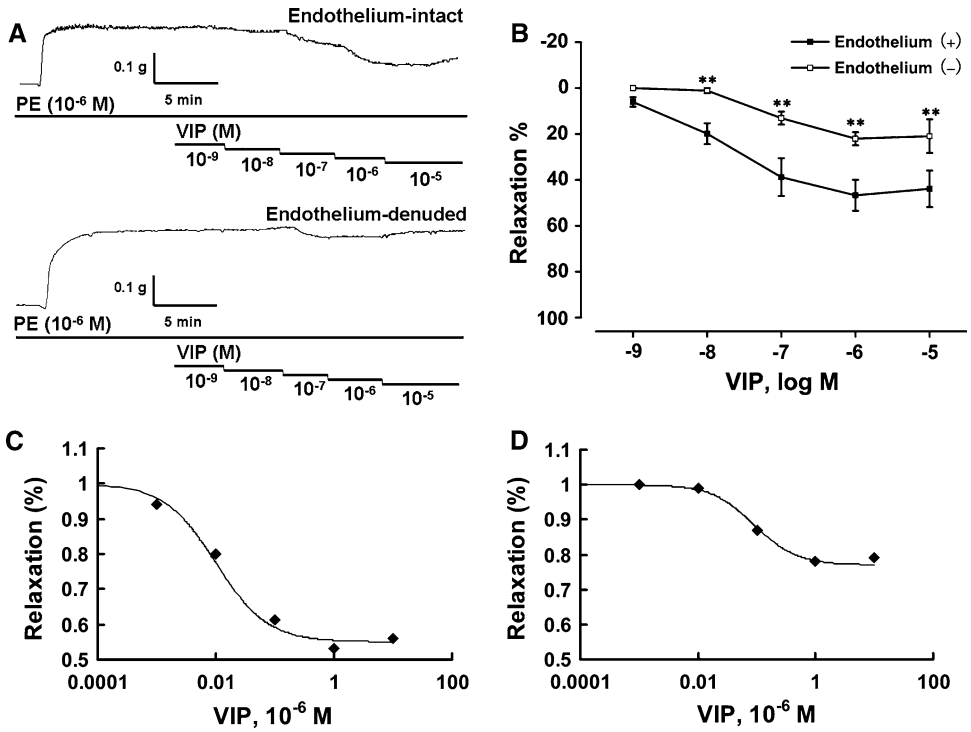

Fig. 1 **a** Concentration-dependent relaxation of EI and ED PA rings induced by VIP (10^{-9} to 10^{-5} M), which was pre-contracted with 10^{-6} M PE. **b** The relationship of VIP versus contractile force in EI (*filled square*, $n = 6$) and ED rings (*open square*, $n = 6$). The response is expressed as a percentage of the active tension originally generated by 10^{-6} M PE. Data are expressed as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ at each point. **c** Based on the data in **b**, the VIP-force relationship of EI rings was described with the Hill equation ($y = 1/(1 + (C/IC_{50})^{h})$, where *y* is normalized force, *C* is VIP concentration, IC_{50} (10^{-8} M) is the VIP concentration at 50% force inhibition, and *h* (1) is the Hill coefficient. **d** Similar data fitting was done in ED rings. The IC_{50} is 9×10^{-8} M and *h* is 1.2

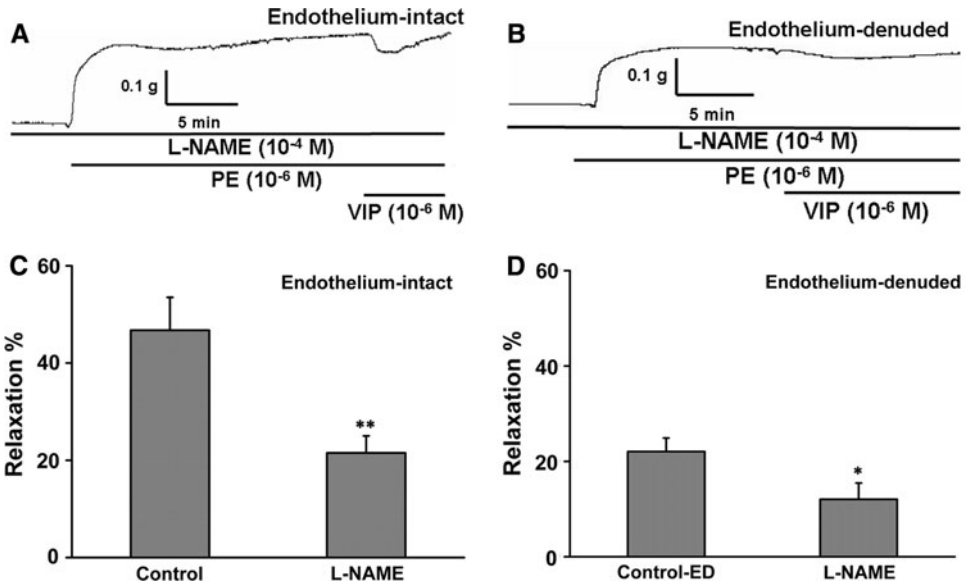

Fig. 2 **a** Representative recording showing the effect of 10^{-6} M VIP on 10^{-6} M PE pre-contracted in the presence of L-NAME in EI rings ($n = 6$). **b** Representative recording showing the effect of 10^{-6} M VIP on 10^{-6} M PE pre-contracted in the presence of L-NAME in ED rings ($n = 5$). **c** In comparison to the EI control group, a treatment of the EI rings with 10^{-4} M L-NAME produced significantly smaller relaxation. **d** In comparison to the control ED group, similarly, treatment with 10^{-4} M L-NAME also diminished relaxation induced by VIP. Responses are expressed as percentage of the active tension originally generated by 10^{-6} M PE. Data were expressed as mean ± SE. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control group

($P < 0.01$, $n = 6$; Fig. 2a, c), suggesting that the NO system in the PA endothelium plays a role in the VIP-induced PA vasorelaxation. In ED rings, similar treatment with L-NAME also decreased vasodilatation of VIP to 12.1 ± 3.4%, significantly different from the control ED rings ($P < 0.05$, $n = 5$; Fig. 2b, d), indicating that a part of vasorelaxation induced by VIP is mediated by VSM.

Relaxation induced by VIP through the PI3K/Akt signaling pathway

Subsequently, we examined the potential involvement of eNOS activity. PA rings with and without endothelium were pretreated with the PI3K/Akt inhibitors wortmannin (10^{-7} M) and LY294002 (3×10^{-5} M), respectively. Then, the vascular response to 10^{-6} M VIP was studied after a pre-contraction with PE. In EI rings the VIP-induced relaxation was diminished markedly after a treatment with wortmannin (12.9 ± 3.1%, $n = 6$) or LY294002 (13.0 ± 4.2%, $n = 6$), both of which were significantly lower than the control rings ($P < 0.01$, Fig. 3a–c). In contrast, similar treatments with these PI3K/Akt inhibitors had no significant effect on the VIP-induced relaxation of ED rings ($P > 0.05$, Fig. 3d).

Western blot analysis

VIP may affect the eNOS by up-regulating its expression and/or stimulating its activity. To address these issues, we performed the Western blot analysis in cultured bovine PAECs. No detectable changes in the eNOS expression were found after a treatment of the PA rings with 10^{-6} M VIP for 5-7 min based on four experiments.

It is known that the eNOS activity is enhanced with phosphorylation at Ser1177. Thus, we examined the eNOS phosphorylation using specific phospho-Ser1177 antibodies. In the experiments, cultured bovine PAECs were treated with 10^{-6} M VIP for 5–7 min before cell lysis. Then protein extracts were subjected to the primary antibodies followed by the secondary antibodies. A ~140-Da band was positively labeled, consistent with the size of total eNOS. This band became stronger after the VIP treatment (Fig. 4a). Similar results were obtained from four experiments. Quantitatively, the VIP treatment enhanced the phosphorylated eNOS by 1.99 times, which was significantly different from the control group ($P < 0.05$, $n = 4$; Fig. 4b). These results suggest that the endothelial PI3K-Akt-eNOS pathway is likely to underscore the VIP relaxation of PA rings.

Activation of the PKA-K_{ATP} channel in VSM was necessary for VIP-induced relaxation

Previous study has shown that K_{ATP} channels in vascular VSM are activated by VIP, leading to depolarization and vasodilatation of MAs [12]. Thus, it is possible that the K_{ATP} channels in the PA VSM are also subject to such a modulation. To test this hypothesis, we treated the ED rings with glibenclamide, a specific K_{ATP} channel blocker.

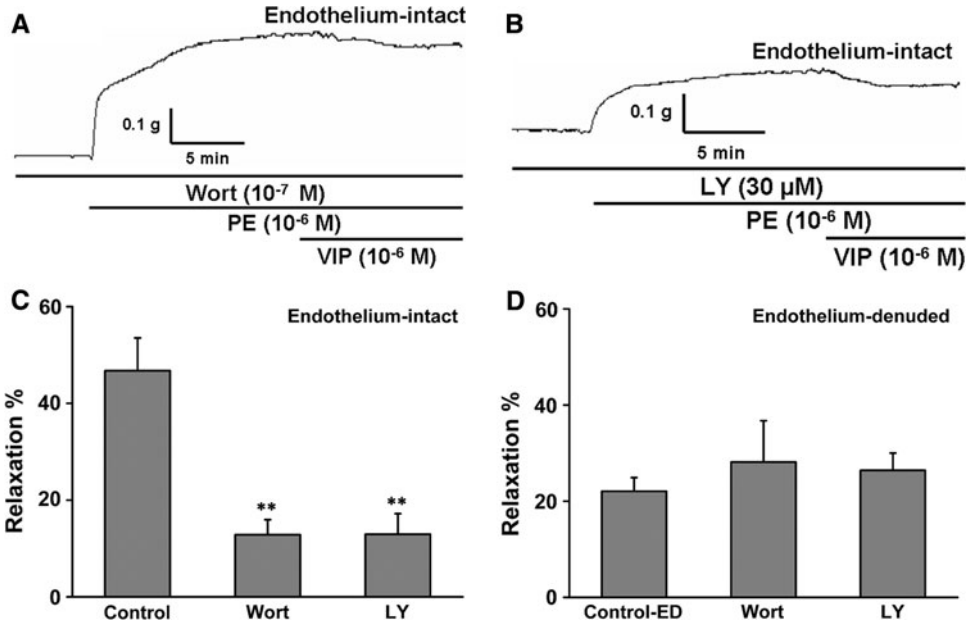

Fig. 3 **a** Representative recording showing the effects of 10^{-6} M VIP on 10^{-6} M PE pre-contracted in the presence of wortmannin (Wort) in EI rings ($n = 6$). **b** Representative recording showing the effect of 10^{-6} M VIP on 10^{-6} M PE pre-contracted in the presence of LY294002 (LY) in EI rings ($n = 6$). **c** In comparison to control, a treatment of the EI rings with either wortmannin or LY294002 produced significantly smaller relaxations. **d** On the other hand, treatment with wortmannin ($n = 5$) or LY294002 ($n = 9$) did not produce any significant relaxation in ED rings compared to control ED. Responses are expressed as percentage of the active tension originally generated by 10^{-6} M PE. Data were expressed as mean ± SE. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control group

An exposure to 10^{-5} M glibenclamide greatly reduced the PA relaxation effect of VIP (9.6 ± 2.3%, $P < 0.01$, $n = 10$; Fig. 5a, c), suggesting that the K_{ATP} channels in the PAs are targeted by VIP. Since a vascular isoform of K_{ATP} channels (Kir6.1/SUR2B) is activated by PKA by phosphorylation of the channel protein [12], we studied the PKA signaling pathway in the VIP signaling. A pretreatment of the ED rings with H-89 (5×10^{-7} M), a selective PKA inhibitor when used in sub-micromolar concentrations, almost totally eliminated the VIP-induced relaxation (6.4 ± 2.2%, $P < 0.01$, $n = 9$; Fig. 5b, c), indicating that the PKA-K_{ATP} channel system is attributable to the relaxation of PA VSM induced by VIP.

Discussion

This is the first systematic study of cellular mechanisms for PA relaxation by VIP. Several lines of evidence were obtained at molecular, cellular and tissue levels, showing the involvement of both the VSM and the endothelium as well as distinct signaling pathways in these cells. The PI3K-Akt-eNOS pathway plays a critical role in the endothelium, whereas the PKA-K_{ATP} channel system is likely to be targeted in the VSM.

Previous studies have suggested the involvement of endothelium in VIP-induced vasorelaxation, although they are not always consistent [13, 14, 17, 18]. The inconsistency may be due to different tissues used in the previous studies [19]. In PAs, our experiments have shown that VIP produces concentration-dependent relaxations. Removal of the vascular endothelium significantly attenuated, though did not abolish, the PA relaxation induced by VIP, indicating that the vasodilatation effects of VIP require the functional integrity of the endothelium.

The endothelial production of NO is tightly regulated at the level of its synthesis through the oxidation of L-arginine by NO synthases (NOSs), a family of three isoenzymes with overlapping patterns of expression. The NOS3 (eNOS) is expressed in vascular endothelial cells throughout the body [20] and is the predominant source of NO production in the pulmonary circulation [21]. The eNOS is known to be regulated by endothelial PI3 K/Akt signaling and the consequent phosphorylation of eNOS in endothelial cells of human umbilical veins and rat lung vasculatures [22–24]. As shown in our studies, blockade of the signaling pathway by the PI3K/Akt inhibitors, wortmannin and LY294002, or the eNOS inhibitor L-NAME markedly eliminated the contribution of endothelium to the relaxation effect of VIP in EI rings. These findings support that such an intracellular signaling is also activated by VIP in PA endothelium. Another striking finding of this work is that VIP elevated phosphorylation of Ser^{1177}-eNOS in bovine PAECs. These data demonstrate that the PI3K/Akt-eNOS

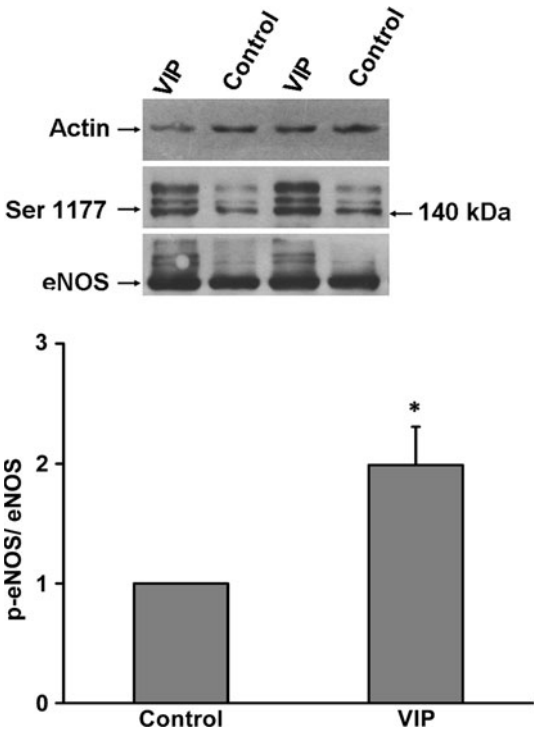

Fig. 4 **a** Bovine PAECs were pre-treated without (control) or with 10^{-6} M VIP for 5–7 min. Lysates were analyzed by Western blot with antibodies to p-eNOS-Ser^{1177} and eNOS. The panel included the same experiment twice on the left and on the right. **b** Phosphorylated Ser1177 eNOS was significantly increased in the VIP-treated group compared to the control group. Bovine PAECs were treated with 10^{-6} M VIP for 5–7 min. Data represent mean ± SE, where control groups were normalized as one from four experiments. *$P < 0.05$ versus control group (0 M VIP)

signal transduction pathway appears to be the endothelial mechanism for the VIP-induced PA dilation.

Accumulating experimental evidence [25, 26] indicates that the primary targets of VIP in other vasculatures and other tissues are the G-protein-coupled VIP receptors, activation of which leads to activation of the G-protein–adenylyl cyclase-PKA system. A previous study [12] has shown that activation of the PKA system is necessary for the vasodilatation effects of VIP on MAs. In airway VSM, the elevated cytosolic cAMP levels activate PKA, which phosphorylates several membrane and/or intracellular proteins, promoting VSM relaxation and bronchodilation [27]. In our present study, the VIP-induced PA dilation is also seen in ED rings, although the magnitude is only half of the EI rings (Fig. 1). The endothelium-independent mechanism in PA VSM has been tackled in the present study, and the results suggest that the downstream target is the K_{ATP} channel (Fig. 5a, c), which is known to play an important role in regulating vascular tones in the systemic arterial system [28]. The current study described that pretreatment with glibenclamide or H-89 strikingly diminished the vasorelaxation response to VIP, which was shown in ED rings, implying that the PKA-K_{ATP} system in rat PA VSM participates in the process of relaxation induced by VIP (Fig. 5). Therefore, our findings in this study extend the understanding of the function of K_{ATP} channels to the PA system.

In conclusion, our current studies indicate that vasodilatation of the rat PA system by VIP involves both endothelium and VSM, in which two distinct intracellular

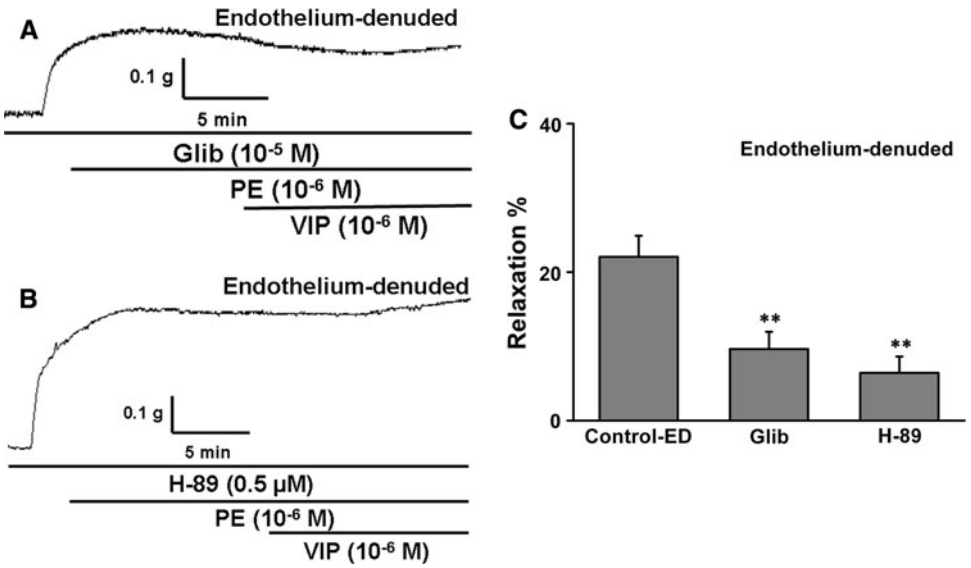

Fig. 5 **a** Representative recording showing the effect of 10^{-6} M VIP on 10^{-6} M PE pre-contracted in the presence of glibenclamide (Glib) in ED rings ($n = 10$). **b** Representative recording showing the effect of 10^{-6} M VIP on 10^{-6} M PE pre-contracted in the presence of H-89 in ED rings ($n = 9$). **c** The ED ring relaxation by 10^{-6} M VIP was significantly reduced by the 30-min pretreatment with 10^{-5} M glibenclamide or 5×10^{-7} M H-89, respectively. Responses are expressed as percentage of the active tension originally generated by 10^{-6} M PE. Data were expressed as mean ± SE. *$P < 0.05$; **$P < 0.01$ versus control group

signaling systems are targeted: i.e., the PKA-K_{ATP} system is a downstream target of VIP signaling in VSM, whereas the PI3K/Akt-eNOS signaling pathway is a downstream target of VIP in the endothelium. Although the involvement of each individual pathway has been suggested in several previous studies [29–32], this current study at the organ, cell and protein levels provides, to our knowledge, novel experimental evidence of the signaling pathways. The demonstration of the VIP effects in PA and its signaling pathways should have impact on the understanding of pulmonary circulation.

Acknowledgments This work was supported by grants from the National Natural Science Foundation of China (nos. 30370578 and 30470752) and the Innovation Foundation of Harbin Medical University (no. HCXS2008009).

References

1. Berkowitz DS, Coyne NG (2003) Understanding primary pulmonary hypertension. Crit Care Nurs Q 26:28–34
2. Farber HW, Loscalzo J (2004) Pulmonary arterial hypertension. N Engl J Med 351:1655–1665
3. Christman BW, McPherson CD, Newman JH, King GA, Bernard GR, Groves BM, Loyd JE (1992) An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. N Engl J Med 327:70–75
4. Giaid A, Saleh D (1995) Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. N Engl J Med 333:214–221
5. Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP, Stewart DJ (1993) Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. N Engl J Med Jun 328:1732–1739
6. Friedman R, Mears JG, Barst RJ (1997) Continuous infusion of prostacyclin normalizes plasma markers of endothelial cell injury and platelet aggregation in primary pulmonary hypertension. Circulation 96:2782–2784
7. Wharton J, Davie N, Upton PD, Yacoub MH, Polak JM, Morrell NW (2000) Prostacyclin analogues differentially inhibit growth of distal and proximal human pulmonary artery smooth muscle cells. Circulation 102:3130–3136
8. Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, Groves BM, Tapson VF, Bourge RC, Brundage BH (1996) A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. The Primary Pulmonary Hypertension Study Group. N Engl J 334:296–302
9. Hoeper MM, Schwarze M, Ehlerding S, Adler-Schuermeyer A, Spiekerkoetter E, Niedermeyer J, Hamm M, Fabel H (2000) Long-term treatment of primary pulmonary hypertension with aerosolized iloprost, a prostacyclin analogue. N Engl J Med 342: 1866–1870
10. Petkov V, Mosgoeller W, Ziesche R, Raderer M, Stiebellehner L, Vonbank K, Funk GC, Hamilton G, Novotny C, Burian B, Block LH (2003) Vasoactive intestinal peptide as a new drug for treatment of primary pulmonary hypertension. J Clin Invest 111: 1339–1346
11. Groneberg DA, Springer J, Fischer A (2001) Vasoactive intestinal polypeptide as mediator of asthma. Pulm Pharmacol Ther 14:391–401
12. Yang Y, Shi Y, Guo S, Zhang S, Cui N, Shi W, Zhu D, Jiang C (2008) PKA-dependent activation of the vascular smooth muscle isoform of KATP channels by vasoactive intestinal polypeptide and its effect of relaxation of the mesenteric resistance artery. Biochim Biophys Acta 1788:88–96
13. Anaid S, Petkov V, Baykuscheva-Gentscheva T, Hoeger H, Painshipp E, Holzer P, Mosgoeller W (2007) Involvement of endothelial NO in the dilator effect of VIP on rat isolated pulmonary artery. Regul Pept 139:102–108
14. Iwabuchi S, Ono S, Tanita T, Koike K, Fujimura S (1997) Vasoactive intestinal peptide causes nitric oxide-dependent pulmonary vasodilation in isolated rat lung. Respiration 64:54–58
15. Adam LP, Franklin MT, Raff GJ, Hathaway DR (1995) Activation of mitogen-activated protein kinase in porcine carotid arteries. Circ Res 76:183–190
16. Ye H, Bi HR, Lv CL, Tang XB, Zhu DL (2005) 15-Hydroxyeicosatetraenoic acid depressed endothelial nitric oxide synthase activity in pulmonary artery. Acta Physiol Sin 57:612–618
17. Sata T, Misra HP, Kubota E, Said SI (1986) Vasoactive intestinal polypeptide relaxes pulmonary artery by an endothelium-independent mechanism. Peptides 7(Suppl 1):225–227
18. Cardell LO, Hjert O, Uddman R (1997) The induction of nitric oxide-mediated relaxation of human isolated pulmonary arteries by PACAP. Br J Pharmacol 120:1096–1100
19. Thom VSM, Hughes AD, Goldberg P, Martin G, Schachter M, Sever PS (1987) The action of calcitonin gene related peptide and vasoactive intestinal peptide as vasodilators in man in vivo and in vitro. Br J Clin Pharmacol 24:139–144
20. Dudzinski DM, Igarashi J, Greif D, Michel T (2006) The regulation and pharmacology of endothelial nitric oxide synthase. Ann Rev Pharmacol Toxicol 46:235–276
21. Coggins MP, Bloch KD (2007) Nitric oxide in the pulmonary vasculature. Arterioscler Thromb Vasc Biol 27:1877–1885
22. Takahashi S, Mendelsohn ME (2003) Synergistic activation of endothelial nitric-oxide synthase (eNOS) by HSP90 and AKT: Ca^{2+}-independent eNOS activation involves formation of an HSP90-AKT-CaM-bound eNOS complex. J Biol Chem 278: 30821–30827
23. Zeng G, Nystrom FH, Ravichandran LV, Cong LN, Kirby M, Mostowski H, Quon MJ (2000) Roles for insulin receptor, PI3-kinase and AKT in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. Circulation 101:1539–1545
24. Hisamoto K, Ohmichi M, Kurachi H, Hayakawa J, Kanda Y, Nishio Y, Adachi K, Tasaka K, Miyoshi E, Fujiwara N (2001) Estrogen induces the AKT-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. J Biol Chem 276:3459–3467
25. Harmar AJ, Sheward WJ, Morrison CF, Waser B, Gugger M, Reubi JC (2004) Distribution of the VPAC2 receptor in peripheral tissues of the mousse. Endocrinology 145:1203–1210
26. Hernandez M, Barahona MV, Recio P, Benedito S, Martinez AC, Rivera L, Garcia-Sacristan A, Prieto D, Orensanz LM (2006) Neuronal and smooth muscle receptors involved in the PACAP- and VIP-induced relaxations of the pig urinary bladder neck. Br J Pharmacol 149:100–109
27. Thirstrup S (2000) Control of airway smooth muscle tone: II-pharmacology of relaxation. Respir Med 94:519–528
28. Quayle JM, Nelson MT, Standen NB (1997) ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. Physiol Rev 77:1165–1232
29. Stojic D, Pesic S, Radenkovic M, Pesic S, Radenkovic M, Popovic-Roganovic J, Pesic Z, Grbovic L (2007) Responses of the human submandibular artery to Ach and VIP. J Dent Res 86(6): 565–570

30. Moore RM, Sedrish SA, Holmes EP, Koch CE, Venugopal CS (2005) Role of endothelium and nitric oxide in modulating in vitro responses of colonic arterial and venous rings to vasodilatory neuropeptides in horses. Can J Vet Res 69:116–122
31. Matsuda NM, Miller SM (2009) Non-adrenergic non-cholinergic inhibition of gastrointestinal smooth muscle and its intracellular mechanism(s). Fundam Clin Pharmacol [Epub ahead of print]
32. Grider JR, Murthy KS (2008) Autoinhibition of endothelial nitric oxide synthase (eNOS) in gut smooth muscle by nitric oxide. Regul Pept 151(1–3):75–79