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Biochemical Pharmacology 65 (2003) 397–405

Biochemical Pharmacology

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Endothelium-dependent and -independent relaxation and VASP serines 157/239 phosphorylation by cyclic nucleotide-elevating vasodilators in rat aorta

Andreas Schäfer[b], Mick Burkhardt[c], Tobias Vollkommer[c], Johann Bauersachs[b], Thomas Münzel[d], Ulrich Walter[c], Albert Smolenski[a,*]

[a]*Institute of Biochemistry II, Faculty of Medicine, University of Frankfurt, Theodor-Stern-Kai 7, D-60590 Frankfurt, Germany*
[b]*Medical Clinic, University of Würzburg, Joseph-Schneider-Str. 2, D-97080 Würzburg, Germany*
[c]*Institute of Clinical Biochemistry and Pathobiochemistry, University of Würzburg, Joseph-Schneider-Str. 2, D-97080 Würzburg, Germany*
[d]*Division of Cardiology, University Hospital Eppendorf, Martinistr. 52, D-20246 Hamburg, Germany*

Received 15 August 2002; accepted 16 October 2002

Abstract

Endothelium-dependent vasodilation is thought to be mediated primarily by the NO/cGMP signaling pathway whereas cAMP-elevating vasodilators are considered to act independent of the endothelial cell layer. However, recent functional data suggest that cAMP-elevating vasodilators such as β-receptor agonists, adenosine or forskolin may also be endothelium-dependent. Here we used functional and biochemical assays to analyze endothelium-dependent, cGMP- and cAMP-mediated signaling in rat aorta. Acetylcholine and sodium nitroprusside (SNP) induced a concentration-dependent relaxation of phenylephrine-precontracted aorta. This response was reflected by the phosphorylation of the vasodilator-stimulated phosphoprotein (VASP), a validated substrate of cGMP- and cAMP-dependent protein kinases (cGK, cAK), on Ser^{157} and Ser^{239}. As expected, the effects of acetylcholine were endothelium-dependent. However, relaxation induced by the β-receptor agonist isoproterenol was also almost completely impaired after endothelial denudation. At the biochemical level, acetylcholine- and isoproterenol-evoked cGK and cAK activation, respectively, as measured by VASP Ser^{239} and Ser^{157} phosphorylation, was strongly diminished. Furthermore, the effects of isoproterenol were repressed by eNOS inhibition when endothelium was present. We also observed that the relaxing and biochemical effects of forskolin were at least partially endothelium-dependent. We conclude that cAMP-elevating vasodilators, i.e. isoproterenol and to a lesser extent also forskolin, induce vasodilation and concomitant cyclic nucleotide protein kinase activation in the vessel wall in an endothelium-dependent way.

Keywords: Endothelium; cGMP; cAMP; Protein kinase; VASP; Isoproterenol

1. Introduction

Vascular tone can be regulated by a variety of vasoconstricting and vasodilating agents. Two major vasodilator pathways utilize the cyclic nucleotides cAMP and cGMP as messenger molecules. The cAMP pathway is initiated by receptor-mediated stimulation of adenylyl cyclase and leads to the activation of cAMP-dependent protein kinase (cAK). cGMP, which is synthesized in the smooth muscle cell layer following the activation of soluble guanylyl cyclase by endothelium-derived nitric oxide (NO), regulates ion-channels, phosphodiesterases and cGMP-dependent protein kinases (cGK) [1]. Type I cGK has been shown to mediate the vasodilatory action of NO in mouse aorta [2], at least at low concentrations of NO [3]. A validated substrate of cGK I as well as cAK is the vasodilator-stimulated phosphoprotein (VASP), a protein localized at actin filaments, focal adhesions and dynamic membrane regions [4]. Other described substrates of cGK I in smooth muscle are the cytoskeletal proteins RhoA [5] and the myosin binding subunit of myosin phosphatase [6]. cGK I also regulates the release of calcium from intracellular stores, e.g. *via* the IP_3-receptor-interacting protein IRAG

* Corresponding author. Tel.: +49-69-6301-5569; fax: +49-69-6301-5895.
E-mail address: smolenski@biochem2.uni-frankfurt.de (A. Smolenski).
Abbreviations: cAK, cAMP-dependent protein kinase; cGK, cGMP-dependent protein kinase; eNOS, endothelial nitric oxide synthase; SNP, sodium nitroprusside; VASP, vasodilator-stimulated phosphoprotein; NO, nitric oxide.

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PII: S0006-2952(02)01523-X

[7]. Usually the NO/cGMP pathway is considered to be endothelium-dependent, whereas cAMP-elevating pathways are not. Recently however, a number of reports have challenged this concept suggesting that cAMP-elevating agents such as β-adrenoreceptor agonists and adenosine have an endothelium-dependent component to the vasorelaxant response [8–12]. One possible mechanism involved could be the activation of eNOS and endothelial NO formation by cAMP [12,13]. A promising new tool for the analysis of cAMP- and cGMP-dependent pathways is the phosphorylation state of the cAK and cGK I substrate VASP. VASP phosphorylation has been shown to be a marker of the activity of the NO/cGMP pathway in a number of studies of animal models of endothelial function and dysfunction [14–18] as well as in humans [19]. These studies were performed using an antibody against the phosphorylation site Ser^{239} of VASP. This antibody was generated by us initially to study cGK I function in platelets [20]. VASP contains two additional cGK phosphorylation sites, namely Ser^{157} and Thr^{278}, although the latter is phosphorylated with low efficiency [21]. In general the VASP Ser^{157} and Ser^{239} sites are phosphorylated by both cAK as well as cGK I, although studies with human platelets and endothelial cells suggested a preference of cGK I for the Ser^{239} site whereas cAK phosphorylates Ser^{157} faster [20,22].

In the present study we determined the extent of endothelium-dependence of cAMP-mediated vasodilation of rat aorta in comparison with the cGMP pathway. In order to gain more information on the mechanisms involved we compared direct activation of adenylyl cyclase using forskolin with indirect receptor-mediated activation of the cAMP pathway using isoproterenol. In addition to vasodilation as common endpoint of the signaling pathways the kinase activity of cAK and cGK was measured directly using VASP Ser^{157} and Ser^{239} phosphorylation.

2. Materials and methods

2.1. Vascular reactivity studies

Male Wistar rats (200–250 g) were purchased from Charles River and anaesthetized with 60 mg/kg pentobarbital administered intraperitoneally (i.p.). The chest was opened and the descending thoracic aorta was dissected, cleaned of connective tissue and cut into 3 mm rings which were mounted in an organ bath (FMI) for isometric force measurements. In some rings endothelium was removed by gentle rubbing of the intimal surface with a stainless steel forceps and only rings with contractile responses to KCl and phenylephrine comparable to endothelium-intact rings were included in the analysis of relaxant responses and VASP phosphorylation. The rings were equilibrated for 30 min under a resting tension of 2 g in oxygenated (95% O_2; 5% CO_2) Krebs–Henseleit solution (NaCl 118 mmol/L, KCl 4.7 mmol/L, $MgSO_4$ 1.2 mmol/L, $CaCl_2$ 1.6 mmol/L, K_2HPO_4 1.2 mmol/L, $NaHCO_3$ 25 mmol/L, glucose 12 mmol/L; pH 7.4, 37 °) containing diclofenac (1 μmol/L) [23]. Rings were repeatedly contracted by KCl (with a maximum of 100 mmol/L) until reproducible responses were obtained. Presence or absence of functional endothelium was confirmed using acetylcholine (1 μmol/L). After 60 min of resting at baseline tension following repeated washouts rings were precontracted with phenylephrine (0.3–1 μmol/L) to comparable constriction levels and the relaxant responses to single doses of the different vasodilators were assessed.

After achievement of stabile relaxant responses the rings were rapidly dismounted and immediately frozen in liquid nitrogen. Afterwards samples were stored at −80 ° until Western blot analysis was performed.

2.2. Western blot analysis

Following vascular reactivity studies frozen tissue (about 2 mg wet weight, 2–3 mm in length) was broken to powder under liquid nitrogen and thawed in 20 μL of lysis buffer containing 20 mM Tris pH 7.5, 250 mM sucrose, 3 mM EGTA, 20 mM EDTA, 1 μM cantharidin, 50 mM pyrophosphate, 1% Triton X-100 and a mix of protease inhibitors (Roche protease inhibitor cocktail, Roche). The sample was incubated on ice for 30 min with occasional mixing and then centrifuged for 10 min at 3000 *g* and 4 °. The supernatant was mixed with 10 μL of 3× SDS sample buffer (200 mM Tris pH 6.7, 6% SDS, 15% glycerol, bromphenolblue and 1/10 vol. β-mercaptoethanol, freshly added) and boiled for 5 min Samples were separated on 9% SDS–PAGE gels and blotted onto nitrocellulose. Blots were cut into half at the position of the 65 kDa proteins, blocked in PBS containing 5% milk powder for 1 hr or overnight and then incubated with primary antibodies in PBS, 5% milk powder, 0.1% Tween 20 for 1 hr at RT. Then blots were washed three times with PBS, 0.1% Tween 20 and incubated with fluorescent-labeled secondary antibodies in PBS, 5% milk powder, 0.1% Tween 20 for 1 hr. After three washes with PBS, 0.1% Tween 20 and one wash with PBS alone bound antibodies were detected and quantified with the Odyssey™ Infrared Imaging System (LI-COR). With this technique two antigens can be detected simultaneously on the same blot with very good signal separation.

2.3. Antibodies

The following primary antibodies were used: rabbit anti-cGK I (1:5000, [24]), mouse anti-α-actinin (1:1000, Sigma), rabbit anti-total-VASP (M4, 1:3000, [25]), mouse anti-phospho-Ser^{239}-VASP (16C2, 2 μg/mL, [20]) and mouse anti-phospho-Ser^{157}-VASP (5C6, 4 μg/mL, [20,22,26]). The secondary antibodies were: Cye 5.5 anti-mouse (1:5000) and IRDye800 anti-rabbit (1:5000), both from LI-COR.

2.4. Substances

Phenylephrine, acetylcholine, sodiumnitroprusside (SNP), forskolin, isoproterenol, N^G-nitro-L-arginine and diclofenac were purchased from Sigma.

2.5. Statistics

Statistical analysis was performed by a two-way ANOVA followed by a Bonferroni *t* test, with probability values of <0.05 considered statistically significant.

3. Results

3.1. Measurement of VASP phosphorylation in rat aorta

We studied the activity of cAMP- and cGMP-regulated protein kinases by measuring the phosphorylation state of their substrate VASP. VASP content and phosphorylation state were studied in Western blots using antibodies against native VASP (Fig. 1) and phosphospecific antibodies against the phosphorylation sites Ser^{239} and Ser^{157}. The phospho-Ser^{157} antibody is a newly developed antibody which was tested in this study for the first time on vascular tissue samples. In rat aorta this antibody detected a single band of 50 kDa (Fig. 1B). To control for total protein loading the amount of α-actinin was determined on the same blot. In addition cGK I expression was measured (Fig. 1). The total amount of cGK I detectable by Western blot was not changed after removal of the endothelium, however about 20% of total VASP was lost. In the presence of endothelium a basal phosphorylation of VASP Ser^{157} and Ser^{239} could be detected ([14] and data not shown). After endothelial denudation the P-Ser^{157} and P-Ser^{239} signals were reduced by 40 and 85%, respectively (Fig. 2). Thus the P-Ser^{239} signal showed the most pronounced endothelium-dependence.

3.2. Effects of acetylcholine and SNP on aortic relaxation and VASP phosphorylation

In phenylephrine-precontracted aortic rings, single doses of acetylcholine induced a concentration-dependent relaxation (Fig. 3A). As expected this response was completely blunted after removal of the endothelium. As shown in Fig. 3B the phosphorylation of both Ser^{157} and Ser^{239} phosphorylation sites of VASP correlated well with the relaxation induced by increasing concentrations of acetylcholine. Both phosphorylation sites were phosphorylated equally well with a maximal phospho-VASP to total-VASP ratio of about 0.35. After removal of the endothelium a slight background phosphorylation remained, which was higher for the Ser^{157} site compared to the Ser^{239} site. The NO-donor sodiumnitroprusside elicited a strong vasodilatory response, which was endothelium-independent (Fig. 4A). The phosphorylation of VASP reflected the rise in vasodilator-activity: the phospho-VASP to VASP ratio reached a maximum of 0.7 at the highest concentration of SNP used (Fig. 4B). Moreover VASP phosphorylation was

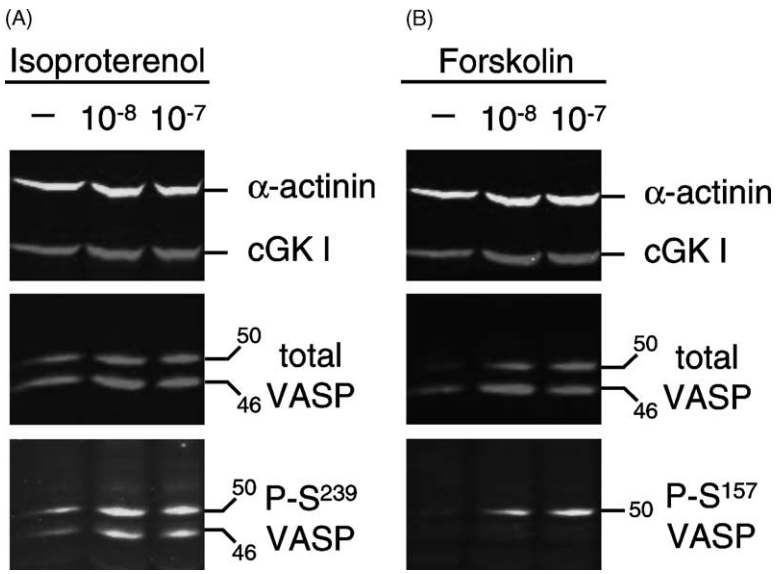

Fig. 1. Expression of cGK and VASP and phosphorylated VASP in intact rat aorta. Rat aortic rings precontracted with phenylephrine were incubated with increasing concentrations (10^{-8} to 10^{-7} M) of isoproterenol in the presence of endothelium (panel A) or forskolin in the absence of endothelium (panel B). After achievement of stabile vasorelaxant responses tissue was frozen, homogenates were prepared and analyzed on Western blots. The blots were cut into half at about the position of the 60 kDa proteins and the lower halves were labeled with specific antibodies for either total VASP (middle blots in panels A and B; lower band: 46 kDa, upper band: 50 kDa) and VASP phosphorylated on serine 239 (lower blot, panel A; lower band: 46 kDa, upper band: 50 kDa) or VASP phosphorylated on serine 157 (lower blot, panel B; 50 kDa). The upper half of the blot was labeled with specific antibodies for cGK I (upper blots on panels A and B; 76 kDa) and α-actinin (upper blots on panels A and B; 100 kDa). Primary antibodies were detected using fluorescence-labeled secondary antibodies and the Odyssey™ Infrared Imaging System and software from LI-COR.

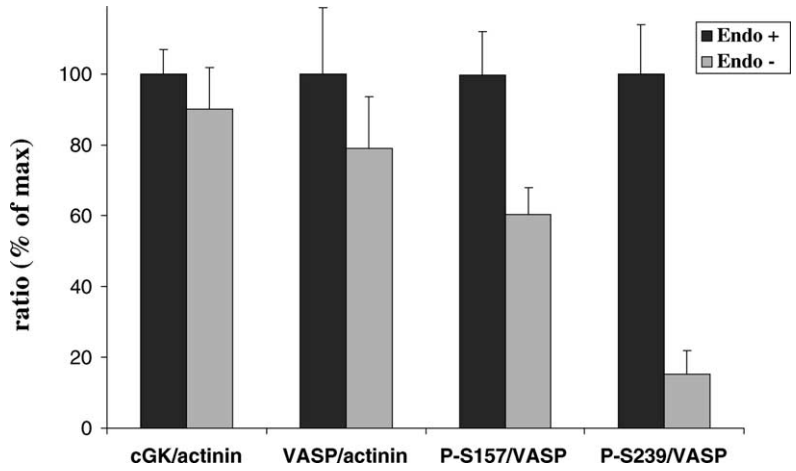

Fig. 2. Effects of endothelial denudation on levels of cGK I, VASP and phospho-VASP in rat aorta. Aortic rings either in the presence or absence of endothelium were contracted with phenylephrine in the presence of diclofenac, then homogenized and analyzed as described in the legend to Fig. 1. cGK I and VASP expression are presented in relationship to the protein standard α-actinin. The phospho-VASP signals are shown as phospho-VASP to total VASP ratios. Dark/light bars represent presence or absence of endothelium, respectively. The means in the presence of endothelium were set to 100% for each protein studied. Data shown are mean ± SEM of 4–10 separate experiments.

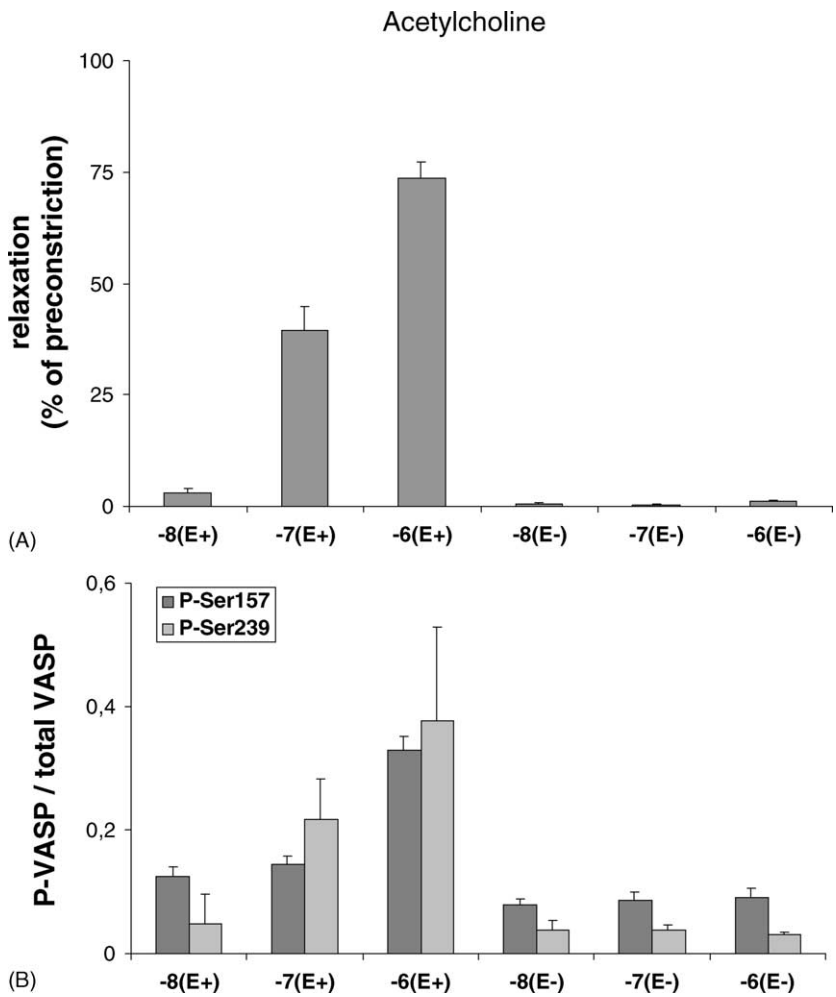

Fig. 3. Effects of acetylcholine on vascular tone and VASP phosphorylation. Precontracted aortic rings were incubated with increasing concentrations of acetylcholine (10^{-8} to 10^{-6} M) in the presence (E+) or absence (E−) of endothelium. In panel A relaxations are shown relative to the precontraction achieved by phenylephrine (1 μM). In the same samples VASP phosphorylation was detected as described in the legend to Fig. 1. VASP phosphorylation was expressed in panel B as ratio of phospho-VASP to total VASP (dark bars = Ser^{157} phosphorylation, light bars = Ser^{239} phosphorylation). Acetylcholine induced a concentration-dependent vasodilation, which was absent after removal of the endothelium. VASP phosphorylation correlated with this effect. Data shown are mean ± SEM of 10–14 (A) and 2 (B) separate experiments.

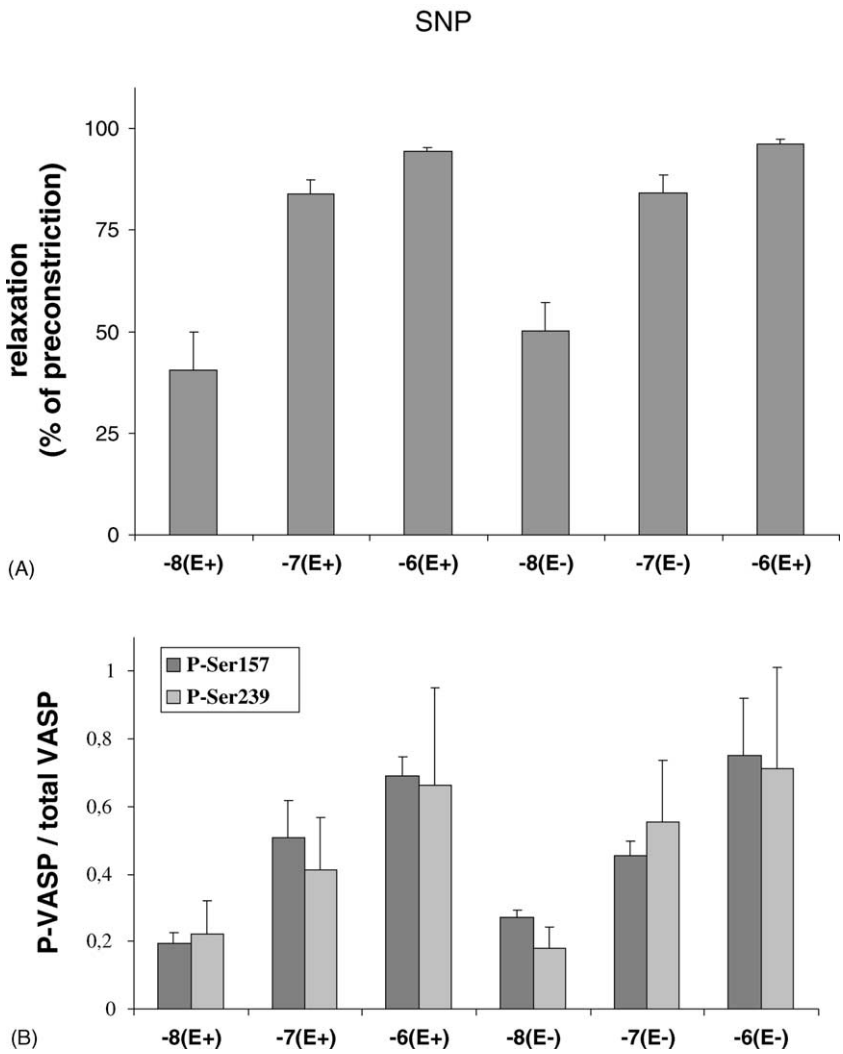

Fig. 4. Effects of SNP on vascular tone and VASP phosphorylation. Precontracted aortic rings were incubated with increasing concentrations of SNP (10^{-8} to 10^{-6} M) in the presence (E+) or absence (E−) of endothelium. In panel A relaxations are shown relative to the precontraction achieved by phenylephrine (1 μM) and a reduction to baseline levels was set as 100%. In the same samples VASP phosphorylation was detected as described in the legends to Figs. 1 and 3. Increasing concentrations of SNP induced increasing degrees of relaxation and VASP phosphorylation. Endothelial denudation did not cause any significant changes. Data shown are mean ± SEM of 9–13 (A) and 2 (B) separate experiments.

independent of the presence or absence of endothelium (Fig. 4B). The intensity of the phospho-VASP signal induced by SNP was similar on both serines studied. cGK I expression was not changed under all conditions tested (data not shown).

3.3. Effects of forskolin and isoproterenol on aortic relaxation and VASP phosphorylation

We next treated phenylephrine-precontracted aortic rings with cAMP-elevating vasodilators. Forskolin generated a partially endothelium-dependent vasodilatory response: the extent of vascular relaxation was reduced in the absence of endothelium only at the lower concentrations of forskolin (10, 100 nmol/L) used, the maximal relaxation at 1 μM/L forskolin was not changed (Fig. 5A). Interestingly the forskolin-induced VASP phosphorylation on Ser^{239} was strongly reduced after endothelial denudation but VASP Ser^{157} phosphorylation remained almost unchanged (Fig. 5B).

In contrast isoproterenol elicited a very striking endothelium-dependent vasodilation (Fig. 6A). This effect was mirrored in the VASP phosphorylation state: after removal of the endothelium only a very weak phospho-VASP signal could be detected with either of the phospho-specific antibodies (Fig. 6B). Interestingly at low isoproterenol concentrations in the presence of endothelium the phosphorylation of the Ser^{239} site appeared much stronger compared to the Ser^{157} phosphorylation. cGK I expression was not changed under the conditions tested (data not shown).

To analyze the role of NO formation for the observed endothelium-dependent effects of isoproterenol we preincubated aortic rings with the NOS inhibitor N^G-nitro-L-arginine (100 μmol/L for 45 min) in the absence of diclofenac. In parallel to endothelium-denuded segments, the

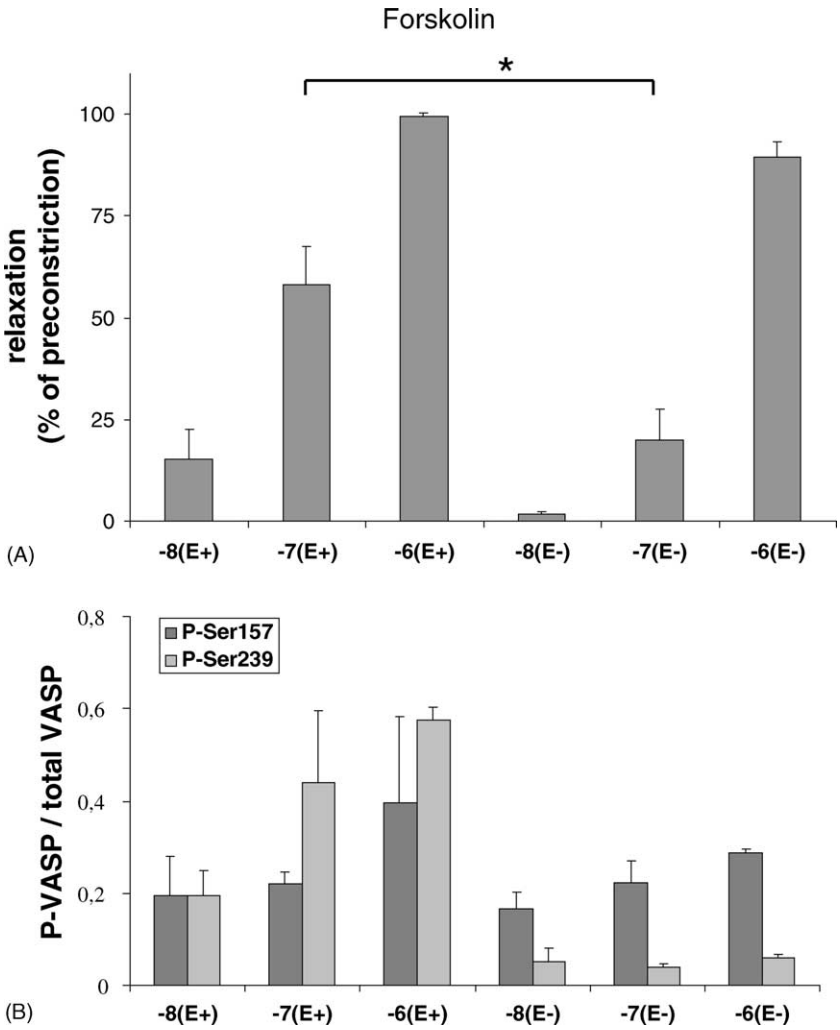

Fig. 5. Effects of forskolin on vascular tone and VASP phosphorylation. Precontracted aortic rings were incubated with increasing concentrations of forskolin (10^{-8} to 10^{-6} M) in the presence (E+) or absence (E−) of endothelium. Vascular relaxation and VASP phosphorylation were measured as described in the legends to Figs. 1 and 3. Forskolin induced a concentration-dependent vasodilation and VASP phosphorylation in the presence of endothelium. After removal of the endothelium forskolin-mediated relaxations were reduced at low concentrations of forskolin used. VASP serine 157 phosphorylation was also reduced, whereas VASP serine 239 phosphorylation was absent. Data shown are mean ± SEM of 7–12 (A) and 2 (B) separate experiments. *Significantly different at $P < 0.001$.

isoproterenol-induced vasodilation was strongly diminished: only one-fourth of the relaxation was achieved with 1 μM isoproterenol in the presence of N^G-nitro-L-arginine as compared to control (data not shown). The phospho-Ser^{239} to total VASP ratio was reduced to 0.15 in the presence of N^G-nitro-L-arginine as compared to 0.5 with isoproterenol alone (data not shown).

4. Discussion

The most important finding of the present study is the pronounced endothelium-dependence of cAMP-elevating vasodilators. Especially isoproterenol-induced vasodilation and cyclic nucleotide regulated kinase activation were almost completely blocked after endothelial denudation. In this respect isoproterenol effects were very similar to the classic endothelium-dependent vasodilator acetylcholine. Furthermore the effect of the direct adenylyl cyclase activator forskolin was at least partially endothelium-dependent. Interestingly forskolin-induced VASP Ser^{239} phosphorylation was completely blocked after removal of the endothelium indicating a loss of cGK I activity. Our functional data are supported by previous studies [8,10,11]. Very recently, Zhang and Hintze [12] reported an increase in nitrite formation in dog coronary microvessels after administration of similar concentrations of isoproterenol and forskolin as used in our study. However, in contrast to this study and our results with isoproterenol, we observed only partially endothelium-dependent effects of forskolin in rat aorta. The differences in extent of cAMP-mediated crossactivation of the NO/cGMP pathway might be explained by species and vessel type differences. One basic mechanism involved in the crosstalk probably is an activation of endothelial NO synthase (eNOS) by the cAMP pathway. eNOS is known as a Ca^{2+}/calmodulin

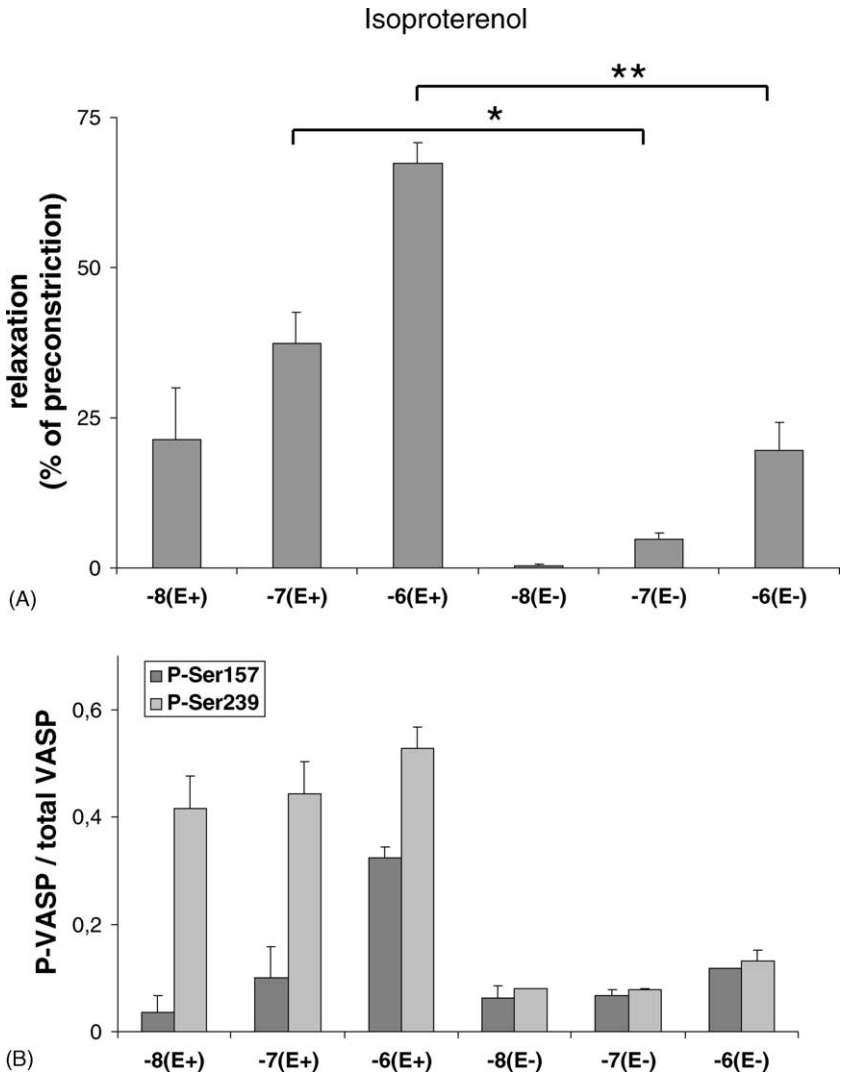

Fig. 6. Effects of isoproterenol on vascular tone and VASP phosphorylation. Precontracted aortic rings were incubated with increasing concentrations of isoproterenol (10^{-8} to 10^{-6} M) in the presence (E+) or absence (E−) of endothelium. Vascular relaxation and VASP phosphorylation were measured as described in the legends to Figs. 1 and 3. Increasing concentrations of isoproterenol-induced increasing relaxation and VASP phosphorylation. Endothelial denudation almost abolished isoproterenol-mediated vasodilations and accordingly VASP phosphorylation was strongly reduced. Data shown are mean ± SEM of 6–10 (A) and 2 (B) separate experiments. *Significantly different at $P < 0.01$. **Significantly different at $P < 0.001$.

(CaM) dependent protein which can be activated by Ca^{2+} mobilizing agents such as acetylcholine or bradykinin. The activation has been suggested to involve a dephosphorylation of eNOS at Thr^{495} and phosphorylation at Ser^{1179} [27–29]. Ser^{1179} of eNOS can be phosphorylated by multiple protein kinases, including AMP-activated protein kinase [30], PKB (serine/threonine protein kinase Akt [31,32]) and also cAK [13]. Boo *et al*. recently suggested eNOS Ser^{1179} phosphorylation by cAK to be responsible for shear stress induced eNOS activation [33]. Our data also hint to a possible crosstalk between the β-adrenoreceptor and eNOS upstream of cAMP since the endothelium-dependence was much more pronounced for isoproterenol than for forskolin. The mechanism of such a cAMP-independent crosstalk is unclear. Maybe a hitherto unknown NOS-interacting protein could mediate the effect in analogy to the described eNOS binding protein hsp90 which serves as a scaffold protein facilitating the phosphorylation and activation of eNOS by PKB [34,35]. Results obtained with cardiac myocytes suggest a colocalization of eNOS and the β-adrenoreceptor in caveolae which might be important for the crosstalk between both pathways as well [36,37].

The good correlation observed in our study between VASP phosphorylation and relaxation of the vascular smooth muscle cell layer strengthens the concept, that VASP is an important indicator of cGK I-induced relaxation of smooth muscle cells. Whether VASP phosphorylation actually mediates some aspects of cGK I-induced vasorelexation is not clear. Data from VASP knock-out mice confirmed the originally proposed role for VASP in platelet inhibition but did not reveal a role in NO/cGMP-induced vasorelaxation [38,39]. However, a complete analysis of VASP deficient mice with respect to the vascular function under physiological and pathophysiological conditions is still lacking.

Also, a large number of VASP-related proteins has been described that could substitute for VASP function in knockout animals [4]. The most likely candidate is the VASP-related protein Mena, which shows conservation of both Ser^{157} and Ser^{239} phosphorylation sites and can be phosphorylated by cAK and cGK [22,40].

In order to compare data as different as vascular tone in an organ bath with protein phosphorylation it is very important to be able to perform both studies in the same tissue sample. Here, we established a method using small vessel pieces directly from the organ bath and analyzing the content of four different antigens and their phosphorylation in parallel in one lane of a Western blot. By this method we could study the activity of cyclic nucleotide regulated signaling pathways in the intact vessel wall with and without endothelium. It should be possible to monitor the activity of even more parameters of complex signal transduction pathways. However, this may require additional developments such as protein chips in order to increase the amount of detectable signals at a given time.

Our results clearly corroborate the concept that VASP phosphorylation can be used as a biochemical marker of the activity of cyclic nucleotide mediated vasodilatory pathways in the vessel wall. However, so far only VASP phosphorylation at Ser^{239} has been studied extensively [14–19]. Our present data demonstrate that VASP Ser^{157} phosphorylation is also a well-suited marker with somewhat different specificity which adds to the biochemical analysis of vasorelaxation. After receptor-mediated activation of endothelial nitric oxide synthase (eNOS) by acetylcholine and after direct activation of soluble guanylyl cyclase with the NO-donor SNP both Ser^{239} and Ser^{157} of VASP were phosphorylated in parallel. Furthermore, the established endothelium-dependent and -independent vasorelaxant effects of acetylcholine and SNP, respectively, were clearly reflected by their effects on VASP phosphorylation. The cAMP-elevating vasodilators induced a differential phosphorylation of both VASP sites. Ser^{239} was phosphorylated strongly after application of forskolin. This data seems to contradict the previously shown specificity of cGMP/cGK I towards the Ser^{239} site [20,22]. However we suggest that forskolin-induced phosphorylation of Ser^{239} is a consequence of its endothelium-dependent activity. In the presence of endothelium forskolin might be able to activate the NO-pathway leading to increased activity of cGK and thus strong phosphorylation of VASP-Ser^{239}. Removal of the endothelium abrogates this effect and only VASP-Ser^{157} phosphorylation remains. A similar effect is observed in the isoproterenol-treated samples: in the presence of endothelium a very striking VASP-Ser^{239} phosphorylation occurs (Fig. 1A), possibly *via* activation of the NO/cGMP/cGK pathway. This response is lost after endothelial denudation. We conclude that VASP Ser^{239} phosphorylation is a very sensitive marker of endothelium-dependent vasodilator functions mediated by the NO/cGMP pathway and finally vascular smooth muscle cGK I. The endothelium-independent effect of forskolin on vascular tone after endothelial denudation could be monitored only by VASP Ser^{157} phosphorylation. Thus in the absence of interfering NO/cGMP effects a specific cAK-mediated phosphorylation of VASP on Ser^{157} induced by cAMP is unmasked. Interestingly removal of the endothelium without subsequent vasodilator treatment resulted only in a partial loss of VASP Ser^{157} phosphorylation. This result might be explained by a basal endothelium-independent activity of cAK within the smooth muscle cell layer. In light of the observed differences in the specificities of the VASP phosphorylation sites as markers of cAMP- vs. cGMP-mediated vasodilatory pathways we suggest that in future studies both phosphorylation sites need to be analyzed in parallel.

In summary, our data demonstrate for the first time by both functional and biochemical analysis a strong endothelium-dependence of cAMP-elevating vasodilators in rat aorta. The necessary crossactivation of endothelial NO production by the cAMP pathway might have an important role in the regulation of vascular function.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (SFB355).

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