Vascular Pharmacology 60 (2014) 95–101

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

PKA and Epac activation mediates cAMP-induced vasorelaxation by increasing endothelial NO production

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ARTICLE INFO

Article history:
Received 28 May 2013
Received in revised form 13 January 2014
Accepted 17 January 2014

Keywords:
Cyclic AMP
Protein kinase A
Epac proteins
Endothelium
eNOS

ABSTRACT

Vascular relaxation induced by 3′,5′-cyclic adenosine monophosphate (cAMP) is both endothelium-dependent and endothelium-independent, although the underlying signaling pathways are not fully understood. Aiming to uncover potential mechanisms, we performed contraction–relaxation experiments on endothelium-denuded and intact rat aorta rings and measured NO levels in isolated human endothelial cells using single cell fluorescence imaging. The vasorelaxant effect of forskolin, an adenylyl cyclase activator, was decreased after selective inhibitor of protein kinase A (PKA), a cAMP-activated kinase, or L-NAME, an endothelial nitric oxide synthase (eNOS) inhibitor, only in intact aortic rings. Both selective activation of PKA with 6-Bnz-cAMP and exchange protein directly activated by cAMP (Epac) with 8-pCPT-2′–*O*-Me-cAMP significantly relaxed phenylephrine-induced contractions. The vasorelaxant effect of the Epac activator, but not that of the PKA activator, was reduced by endothelium removal. Forskolin, dibutyryl cAMP (a cAMP analogue), 6-Bnz-cAMP and 8-pCPT-2′–*O*-Me-cAMP increased NO levels in endothelial cells and the forskolin effect was significantly inhibited by inactivation of both Epac and PKA, and eNOS inhibition. Our results indicate that the endothelium-dependent component of forskolin/cAMP-induced vasorelaxation is partially mediated by an increase in endothelial NO release due to an enhanced eNOS activity through PKA and Epac activation in endothelial cells.

1. Introduction

3′,5′-Cyclic adenosine monophosphate (cAMP) is an ubiquitous second messenger that plays an important role in the regulation of vascular tone. Several mechanisms have been proposed to explain its direct, endothelium-independent vasorelaxant effects, including a decrease of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) via different pathways, a reduction of the sensitivity of contractile filaments to $[Ca^{2+}]_c$, or an activation of certain K^+ channel subtypes (for detailed review see, e.g., [1,3,26]). Although for many years it was thought that vascular smooth muscle relaxation induced by cAMP occurred independently from a functional endothelial layer, it is now generally accepted that the endothelium also participates in this process, although the underlying mechanisms are not yet fully understood [14,31,38,45].

It is now established that several of the effects induced by cAMP-elevating agents that were considered to be mediated by the activation of cyclic-AMP protein kinase (PKA) are attributable to the activation of the alternative cAMP target Epac (exchange protein directly activated by cAMP) in different cell types [17,36,39]. Despite the plethora of studies on this subject, the specific roles of PKA and Epac have not been extensively investigated in vascular smooth muscle relaxation [25,39].

In light of these reports and with the aim of further investigating the contribution of the endothelium to the vasorelaxant effects of cAMP, we examined the vasorelaxant effects of forskolin, an adenylyl cyclase activator, on isolated rat aortic rings in the presence and in the absence of a functional endothelium. Previous reports indicate that forskolin significantly elevates $[cAMP]_i$ both in rat aortic myocytes [4,8] and in human endothelial cells [5]. Here, we have investigated a potential role of PKA and Epac in the vasorelaxant effects of forskolin by using selective activators and inhibitors of both proteins. We also studied the role of NO synthesis in cAMP-induced vasorelaxation by inhibiting endothelial NO synthase (eNOS) with the selective inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME; [33]) in rat aortic rings and by performing

Abbreviations: $[Ca^{2+}]_c$, cytoplasmic Ca^{2+} concentration; cAMP, 3′,5′-cyclic adenosine monophosphate; DAF-2, 4,5-diaminofluorescein; DAF-2-DA, 4,5-diaminofluorescein diacetate; db-cAMP, dibutyryl cyclic AMP; DMSO, dimethyl sulfoxide; eNOS, endothelial NO synthase; Epac, exchange protein directly activated by cAMP; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; KBS, Krebs bicarbonate solution; L-NAME, N_ω-nitro-L-arginine methyl ester; PKA, cyclic-AMP protein kinase.

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http://dx.doi.org/10.1016/j.vph.2014.01.004

imaging experiments in isolated human umbilical vein endothelial cells (HUVEC) using 4,5-diaminofluorescein (DAF-2).

2. Materials and methods

2.1. Animals and ethical approval

Male Wistar–Kyoto (WKY) rats (Iffa-Credo) weighing 200–350 g were used throughout this study. They were housed, cared for and acclimatized (before the experiments) as previously indicated [29]. For experiments, rats were killed by CO_2 inhalation and exsanguinated. All experimental protocols were approved by the bioethics committee of the University of Santiago de Compostela (Spain) and the bioethics committee for research (CEIC) of the Xunta de Galicia (Spain).

2.2. Contraction–relaxation studies in isolated rat thoracic aorta rings

For contraction–relaxation experiments, ~4 mm long rat thoracic aortic rings were transferred into an organ bath containing Krebs bicarbonate solution (KBS) at 37 °C (composition in mM: 119 NaCl, 4.7 KCl, 1.5 $CaCl_2 \cdot 2H_2O$, 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 0.03 EDTA-Na_2, 11 glucose; pH 7.4), oxygenated with carbogen (95% O_2 + 5% CO_2), following the protocol previously described [8]. In some rings, the endothelium was removed by gently rubbing the intimal surface with a cotton bud moistened with KBS.

Aortic rings were then equilibrated at a resting tension of 2 g for at least 1 h, during which KBS was replaced every 15 min. Thereafter, a contraction was induced by the addition of phenylephrine (1 μM). Once the contraction stabilized, a single concentration of acetylcholine (1 μM) was added to the bath in order to assess the endothelial integrity of the preparations. The endothelium was considered to be intact when this drug elicited a vasorelaxation >50% of the maximal contraction obtained. In rubbed rings, the absence of endothelium was confirmed by the absence of acetylcholine relaxant actions.

After assessing the presence or absence of functional endothelium, vascular tissues were allowed to recuperate for at least 1 h during which KBS was replaced every 15 min, before any experiment protocol was started.

2.3. Western blot

To investigate Epac-1 and Epac-2 protein expression, rat thoracic aortas were washed in ice-cold Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS), snap-frozen in liquid nitrogen and homogenized in RIPA lysis buffer (composition in mM: 150 NaCl, 50 Tris–HCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS; pH 8.0) supplemented with proteinase inhibitor cocktail. For VASP phosphorylation studies, the aortas were incubated in KBS with vehicle (control) or the appropriate drug for 10 min at 37 °C. Then, the aortas were washed in ice-cold PBS, snap-frozen in liquid nitrogen and homogenized in Tris-Triton lysis buffer (composition in mM: 100 NaCl, 10 Tris–HCl, 1 EDTA, 1 EGTA, 1% Triton X-100, 10% glycerol, 0.5% sodium deoxycholate and 0.1% SDS; pH 7.4) supplemented with proteinase and phosphatase inhibitor cocktail.

Tissue lysates were sonicated and centrifuged at 14,000 *g* for 20 min at 4 °C. Protein concentrations were determined using bicinchoninic acid (BCA) protein assay, according to the manufacturer's protocol.

Western blot experiments were performed as previously described [12]. Samples containing 30 μg of total protein were loaded on an SDS-PAGE gel (8%) for electrophoresis and transferred onto a nitrocellulose membrane according to standard protocols. Subsequently, membranes were blocked for 30 min at room temperature in blocking buffer (3% bovine serum albumin, 0.2% Tween 20 in Tris-buffered solution) and incubated overnight at 4 °C with specific primary antibodies: mouse anti-Epac-1 (1:500), mouse anti-Epac-2 (1:500), mouse anti-GAPDH (1:1000), and rabbit-anti-VASP (1:500, which recognizes both phosphorylated VASP and non-phosphorylated VASP), diluted in blocking buffer, overnight at 4 °C. Membranes were washed and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. Bands were detected by enhanced chemiluminescence using the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). GAPDH was used as a protein loading control.

2.4. NO imaging in isolated HUVEC

Human umbilical vein endothelial cells (HUVEC-C, a spontaneously transformed cell line) were purchased from American Type Culture Collection (ATCC 1730-CRL; Rockville, MD, USA). This cell line is a pure population of cells that preserve established endothelial cell characteristics [16].

Cell culture and NO imaging experiments were performed as previously described in detail [11]. Briefly, the cells were grown in DMEM/F12 medium supplemented with endothelial cell growth supplement (0.03 mg/ml), heparin (0.1 mg/ml), antibiotics/antimycotic (100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B) and 10% (v/v) fetal bovine serum (FBS). Cells were maintained at 37 °C with 5% CO_2 in air and used for experimentation between passages 2 and 15. The passage number did not alter cell response. For imaging experiments, HUVEC were seeded at ~1500 cells/cm^2 in 35 mm glass-bottom Petri dishes (World Precision Instruments Ltd., London, UK) and kept in culture for 48 h before the experiments. Cells were then incubated for 60 min at 37 °C in normal bathing solution (composition in mM: 140 NaCl, 5 KCl, 1.5 $CaCl_2.2H_2O$, 2 $MgCl_2$, 10 HEPES, 11 glucose; pH 7.4) containing L-arginine (100 μM) and DAF-2 diacetate (DAF-2-DA, 5 μM), which penetrates cells, where it is hydrolysed by intracellular esterase activity to DAF-2, a NO-sensitive fluorescent dye [23]. Cells were then gently washed twice with normal bathing solution and allowed to rest for >15 min in the incubator. Cells loaded with DAF-2-DA were placed on an inverted light microscope and excited at 490 ± 10 nm (200 ms exposure time). Emitted fluorescence was collected through a 510 ± 20 nm emission filter and measured with an intensified CCD camera (Rolera XR Monofast 1394 cooled, QImaging, Surrey, Canada). Fluorescent images were generated at 5 s intervals, using 2 averaged images at each wavelength, and digitally stored for later analysis with MetaFluor software (Universal Imaging Corporation, West Chester, PA, USA).

For incubation periods, drugs (or vehicle controls) were added in volumes of 1 to 10 μl to a final incubation volume of 1 ml of bathing solution. In some experiments, the NO donor sodium nitroprusside was used as a positive control for the technique. All procedures and experiments were performed at room temperature to minimize compartmentalization and cell extrusion of the fluorescent dye.

2.5. Data presentation and statistical analysis

Unless otherwise stated, results shown in the text and figures are expressed as mean ± S.E.M. Significant differences between two means ($P < 0.05$ or $P < 0.01$) were determined by Student's two-tailed *t* test for paired or unpaired data or by one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test, where appropriate.

In the experiments carried out in pre-contracted rat aortic rings, relaxant responses are expressed as a percentage of the maximal contraction (E_{max} = 100%) produced by the phenylephrine. In cumulative experiments, sigmoidal concentration–response curves for the vasorelaxant effects of the drugs were fitted using the program Origin™ 7.0 (Microcal Software, Inc., Northampton, USA), with an estimation of IC_{50} values (i.e. concentrations inducing 50% relaxation) for phenylephrine-induced contractions.

In imaging experiments, for each isolated HUVEC, the fluorescence emitted was averaged from pixels within manually outlined cell areas. Background compensation was performed by subtracting the