Cellular Signalling 28 (2016) 109–116

Contents lists available at ScienceDirect

Cellular Signalling

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

Cyclic guanosine monophosphate compartmentation in human vascular smooth muscle cells

Joana Feiteiro, Ignacio Verde, Elisa Cairrão *

CICS-UBI — Centro de Investigação em Ciências da Saúde, University of Beira Interior, Covilhã, Portugal

ARTICLE INFO

Article history:
Received 29 September 2015
Received in revised form 1 December 2015
Accepted 8 December 2015
Available online 9 December 2015

Keywords:
Compartmentalization
Cyclic nucleotide-gated channels
Cyclic GMP
Potassium current
Phosphodiesterases

ABSTRACT

Aims: The role of different vascular subtypes of phosphodiesterases (PDE) in cGMP compartmentalization was evaluated in human smooth muscle cells.
Methods and results: To understand how the cGMP conveys different information we infected smooth muscle cells with adenovirus containing mutants of the rat olfactory cyclic nucleotide-gated (CNG) channel-subunit and we recorded the associated cGMP-gated current (I_{CNG}). The whole cell configuration of patch clamp technique was used to measure the I_{CNG} and also the potassium current (I_K) in human umbilical artery smooth muscle cells (HUASMC). ANP (0.1 μM) induced a clear activation of basal I_{CNG}, whereas SNP (100 μM) had a slight effect. The nonselective PDE inhibitor (IBMX; 100 μM), the PDE5 inhibitor (T0-156; 1 μM) and the PDE3 inhibitor (cilostamide; 10 μM), all had a tiny effects on the basal I_{CNG} current. Concerning potassium channels, we observed that ANP and testosterone induced activation of I_K and this activation is bigger than that elicited by SNP, cilostamide and T0-156. Cilostamide and T0-156 decreased the CNG stimulation induced by ANP and testosterone, suggesting that pGC pool is controlled by PDE3 and 5. Thus, the effects of SNP show the existence of two separated pools, one localized next to the plasma membrane and controlled by the PDE5 and PDE3, and a second pool localized in the cytosol of the cells that is regulated mainly by PDE3.
Conclusions: Our results show the existence of cGMP compartmentalization in human vascular smooth muscle cells and this phenomenon can open new perspectives concerning the examination of PDE families as therapeutic targets.

1. Introduction

CNG channels have been used in the last years to monitor and to define the spatial and temporal changes in cyclic nucleotides in living cells. The CNGA2, translate changes in intracellular cyclic nucleotide concentration in electrical response and/or a sign of Ca^{2+} intracellular because they open by the direct binding of cAMP and cGMP [1].

In living cells, the cGMP synthesis is controlled by two types of guanylyl cyclases that differ in their cellular location and in their activation by specific ligands. The particulate guanylyl cyclases (pGC) localized at the plasma membrane, which is activated by natriuretic peptides such as atrial (ANP), brain and C-type natriuretic peptides [2–4]. The soluble guanylyl cyclase (sGC) is present in the cytosol and is activated by nitric oxide (NO) [4,5]. Although both cyclases synthetise cGMP, the consequences of activating pGC or sGC often lead to dissimilar functional effects. One explanation for these divergent effects is that different stimuli induce cGMP rises in distinct and specific subcellular locations, hence regulating dissimilar targets in different parts of the cells [6–10]. Thus, the increase of cGMP induced by natriuretic peptides and by NO donors may occur in different subcellular compartments [6,7,11].

The existence of subcellular compartments of cyclic nucleotides cAMP was discovered some decades ago [12] and explains different effects observed in cardiomyocytes [13–16]. Some authors suggested that this phenomenon is due to a physical barrier formed by parts of the endoplasmic reticulum located near the plasma membrane [14]. Other authors suggested that this phenomenon depends on the location of various components associated with signal transduction pathways cyclic nucleotide [17]. The cyclic nucleotides localized signals are important to determine the speed and the specificity of events mediated by the cyclic nucleotides. This allows the cells to respond differently to different external stimuli which act on the same second messenger. Thus, changes in cyclic nucleotide concentration in subcellular places may orchestrate a variety of cellular responses [14]. Several years ago, it was demonstrated that PDE, the enzymes degrading cyclic

Abbreviations: ANP, atrial natriuretic peptide; BK_{Ca}, large-conductance Ca^{2+} activated potassium channels; cAMP, cyclic adenosine 3,5′-monophosphate; CNG, cyclic nucleotide-gated; cGMP, cyclic guanosine 3,5′-monophosphate; HUASMC, human umbilical artery smooth muscle cell; I_{CNG}, cyclic nucleotide-gated current; I_K, potassium current; IBMX, 3-Isobutyl-1-methylxanthine; K_V, voltage-gated potassium channels; NO, nitric oxide; PDE, phosphodiesterase; pGC, particulate guanylyl cyclase; PKG, protein kinase G; sGC, soluble guanylyl cyclase; SMC, smooth muscle cells; Sp-8, 8-((4-chlorophenylthio) guanosine-3′ 5′-cyclic monophosphate).

* Corresponding author at: Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal.
E-mail address: ecairrao@fcsaude.ubi.pt (E. Cairrão).

http://dx.doi.org/10.1016/j.cellsig.2015.12.004
0898-6568/

nucleotides, participate in its compartmentalization [13,15,18–20]. Different effects between after stimulation of the ANP and SNP pathways in human umbilical artery smooth muscle cells (HUASMC) concerning the stimulation of potassium currents were also observed [21]. Moreover, Santos-Silva et al. [22] in HUASMC demonstrated that the two major PDE isoforms bind and hydrolyze intracellular cGMP: PDE3, which hydrolyzes preferentially cAMP and is inhibited by cGMP; and PDE5, which is highly specific for cGMP. Our data and that obtained in cardiomyocytes suggest the existence of subcellular compartments of cyclic nucleotides in vascular smooth muscle cells. However, few information was obtained concerning compartmentalization of cyclic nucleotides in vascular smooth muscle cells.

In this study, our aim was to characterize and compare in real time the changes in subsarcolemmal cGMP concentration in response to activation of sGC and pGC. For that purpose, we used the wild-type (WT) α-subunit of the rat olfactory cyclic nucleotide-gated channel (CNGA2) as real-time sensor for subsarcolemmal cGMP [23]. This channel binds cGMP with a >10-fold higher affinity than cAMP [23]. Here we describe experiments performed on HUASMC infected with an adenovirus encoding the WT CNGA2 (Ad-CNGA2). Using this model, we analyzed the effect of atrial natriuretic peptide (ANP, a stimulator of pGC) and sodium nitroprusside (SNP, a stimulator of sGC) and we provide evidence for cGMP compartmentalization and identify PDE3 and PDE5 isoforms as key elements in this phenomenon.

2. Methods

2.1. Tissue preparation

Pieces (3–7 cm) of the umbilical cord were obtained from normal term pregnancies after vaginal delivery with the consent of the donor mothers. All the procedures performed with the umbilical cords in the present study were approved by the Ethics Commol/Littee of Centro Hospitalar da Cova da Beira (Covilhã, Portugal) and conformed to the tenets of the Declaration of Helsinki.

Umbilical cord samples were collected in sterile physiological saline solution (PSS; composition (in mmol/L): NaCl 110; $CaCl_2$ 0.15; KCl 5; $MgCl_2$ 2; HEPES 10; $NaHCO_3$ 10; KH_2PO_4 0.5; NaH_2PO_4 0.5; glucose 10; EDTA 0.49). To avoid contamination and tissue degradation, penicillin (5 U/mL), streptomycin (5 μg/mL), amphotericin B (12.5 nmol/L) and antiproteases (leupeptin 0.45 mmol/L, benzamidine 26 mmol/L and trypsin inhibitor 10 mmol/L) were added to the PSS. Umbilical arteries were isolated from the surrounding connective tissue and cut into 30–50 mm rings. The human umbilical artery (HUA) rings were cut up into rectangular pieces with scissors so that the smooth muscle layers from the tunica media could be retrieved using surgical forceps and a scalpel. The smooth muscle layers were used for subsequent cell dissociation and culture (see below).

2.2. Cell dissociation and culture

Smooth muscle cells were isolated from the HUA as previously described [24]. Briefly, the layers extracted from the tunica media were cut into small fragments and placed in PSS (without antiproteases but containing 1–2 mg/mL collagenase V, 0.3–0.5 mg/mL elastase, 0.6 mg/mL trypsin inhibitor and 1.25 mg/mL taurine) for 10 min at 37 °C. The hydrolytic action of the enzymes was stopped by the addition of Dulbecco's modified Eagle's medium (DMEM)–F12 cell culture medium containing 10% fetal bovine serum (FBS). The digested tissue was subjected to gentle shaking for 10 min, after which it was filtered through a 500 μmol/L mesh. The filtered solution, containing isolated HUA smooth muscle cells, was centrifuged at 150 g for 5 min at 21 °C. The supernatant was discarded and the pellet was suspended in plating medium, consisting of DMEM-F12 containing 5% FBS, 5 μg/mL epidermal growth factor (EGF), 0.5 nmol/L fibroblast growth factor (FGF), 2 μg/mL heparin and 5 μg/mL insulin. Cells were plated in collagen-coated culture dishes at 37 °C under an atmosphere of 95% air and 5% CO_2. The culture medium was changed every 2–3 days and confluent cultures were obtained after 15–20 days. Confluent cells were subcultured until passage 7.

2.3. Infection

The infection of HUASMC with WT CNGA2 encoding adenovirus (Ad-CNGA 2) was performed as previously described [25]. The confluent cultures of HUASMC were placed for 1 h in the FBS-free cell culture medium DMEM-F12. After this time, the medium is replaced by 400 μL of free-FBS medium containing WT CNGA2 encoding adenovirus (Ad-CNGA2), used at a multiplicity of infection (MOI) of 5000 plaque forming units per cell (pfu/cell). The Ad-CNGA2 was a generous gift from Dr. Dermot Cooper (University of Cambridge, United Kingdom).

After infection, the cells are placed in an incubator for 24 h. After this period the medium is replaced by DMEM-F12 containing 5% FBS and placed 12 h in an incubator. After that the cells were used to perform the experiments.

2.4. Immunocytochemistry

The HUASMC attached onto coverslips (1×10^4 cells/dish) were fixed, permeabilized and incubated with a mouse monoclonal antibody against CNGA2 as previously described [13,24]. This antibody was a generous gift of Drs. F. Mueler and B. Kaupp (Juelich, Germany). Cells were revealed with Alexa fluor 488 goat anti-mouse. After mounting the samples, the expressed proteins were localized using a Confocal fluorescence imaging with a confocal laser scanning (Carl Zeiss, LSM510 Meta) with 488 nm laser excitation for the fluorescein isothiocyanate (FITC) channels. A 40× ApoChromat,1.4-NA objective (Carl Zeiss) was used to ensure high-resolution images. DIC images were collected simultaneously with transmitted light by excitation at 633 nm. Images were processed with LSM Image Browser (Zeiss) and Adobe Photoshop.

2.5. Electrophysiology experiments

Cells from the first to the sixth passages were trypsinized and used to perform the electrophysiology experiments.

The CNGA2 current (I_{CNG}) in HUASMC was analyzed in the whole cell configuration of the patch clamp technique. The cells were maintained at 0 mV holding potential and routinely hyperpolarized every 8 s to −50 mV test potential during 200 ms. I_{CNG} was recorded in the absence of divalent cations in the extracellular solutions contains (mmol/L): NaCl 107, CsCl 20, HEPES 10, Glucose 5 and pH 7.4; allowing monovalent cations to flow through the channels in a non-specific manner. Patch electrodes (2.8–4.2 MΩ) were made of soft glass and filled with control internal solution (Reagent 5, Portugal) and filled with control internal solution containing (in mmol/L): HEPES 20, EGTA 20, Glucose 5, TEA 5, CsCl 113.8, $MgCl_2$ 2.5, $CaCl_2$ 0.062, Na_2ATP 3.1, Na_2GTP 0.42, adjusted to pH 7.3 with CsOH. Basal I_{CNG} were measured 5–10 min after patch break to allow the equilibration between pipette and intracellular solutions.

The potassium current (I_K) was analyzed in HUASMC using the amphotericin B-performed whole-cell patch-clamp methodology [21,26]. The cells were maintained at a holding potential of −80 mV and routinely depolarized every 8 s to 60 mV test potential during 350 ms to measure I_K. The external solution contained (mmol/L): NaCl 134.3, $CaCl_2$ 1.0, HEPES 5.0, KCl 5.4 and glucose 6.0, pH 7.4 adjusted with NaOH. The internal solution contained (mmol/L): KCl 125.0, $MgCl_2$ 1.0, Na-ATP 5.0, Na-GTP 0.5, EGTA 0.1, HEPES 20.0 and glucose 10.0, pH 7.3 adjusted with KOH.

Currents I_{CNG} and I_K were not compensated for capacitance and leak currents. All experiments were done at room temperature (21–25 °C)