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Heterodimerization with the β_1 subunit directs the α_2 subunit of nitric oxide-sensitive guanylyl cyclase to calcium-insensitive cell-cell contacts in HEK293 cells: Interaction with Lin7a

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ABSTRACT

Nitric oxide-sensitive guanylyl cyclase is a heterodimeric enzyme consisting of an α and a β subunit. Two different α subunits (α_1 and α_2) give rise to two heterodimeric enzymes α_1/β_1 and α_2/β_1. Both coexist in a wide range of tissues including blood vessels and the lung, but expression of the α_2/β_1 form is generally much lower and approaches levels similar to the α_1/β_1 form in the brain only. In the present paper, we show that the α_2/β_1 form interacts with Lin7a in mouse brain synaptosomes based on co-precipitation analysis. In HEK293 cells, we found that the overexpressed α_2/β_1 form, but not the α_1/β_1 form is directed to calcium-insensitive cell-cell contacts. The isolated PDZ binding motif of an amino-terminally truncated α_2 subunit was sufficient for cell-cell contact localization. For the full length α_2 subunit with the PDZ binding motif this was only the case in the heterodimer configuration with the β_1 subunit, but not as isolated α_2 subunit. We conclude that the PDZ binding motif of the α_2 subunit is only accessible in the heterodimer conformation of the mature nitric oxide-sensitive enzyme. Interaction with Lin7a, a small scaffold protein important for synaptic function and cell polarity, can direct this complex to nectin based cell-cell contacts via MPP3 in HEK293 cells. We conclude that heterodimerization is a prerequisite for further protein-protein interactions that direct the α_2/β_1 form to strategic sites of the cell membrane with adjacent neighbouring cells. Drugs increasing the nitric oxide-sensitivity of this specific form may be particularly effective.

1. Introduction

Nitric oxide-sensitive guanylyl cyclase (NOsGC) is the major physiological receptor for nitric oxide (NO) in cells of the cardiovascular and central nervous system. Two heterodimeric enzymes α_1/β_1 and α_2/β_1 are known to be important for NO-signaling in humans and generate cGMP from GTP. NO-releasing drugs lead to activation of both isoforms, as they share the activation mechanism by NO [1]. The discovery of drugs that activate the enzyme independent of NO allows for a differentiation of isoforms [2]. In fact, there is evidence for a prevalence of the activator drug cinaciguat for the α_1/β_1 form [3]. BAY41-2272, a stimulator drug closely related to riociguat used in pulmonary hypertension, increases the NO-sensitivity of both guanylyl cyclase isoforms [4].

Both heterodimeric enzymes coexist in a wide range of tissues including blood vessels and the lung, but expression of the α_2/β_1 form is generally much lower and approaches similar levels to the α_1/β_1 form in the brain only [5]. A specific role of the α_2/β_1 form in the central nervous system is supported by its interaction with the third PDZ (PSD-95/disc large/zonula occludens) domain of the postsynaptic density scaffold protein 95 (PSD-95) [6]. Despite the small amount of the α_2/β_1 form in the cardiovascular system, this isoform seems surprisingly effective in preventing

Abbreviations: ANOVA, analysis of variance; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; CFTR, cystic fibrosis transmembrane conductance regulator; CFP, cyan fluorescent protein; CLSM, confocal laser scanning microscopy; FLIM, fluorescence-lifetime imaging microscopy; HEK293 cells, human embryonic kidney cells; L27 domain, Lin2-Lin7 domain; MPP3, membrane palmitoylated protein 3; NMRI, Naval Medical Research Institute; NOsGC, nitric oxide-sensitive guanylyl cyclase(s); PDZ, PSD-95/disc large/zonula occludens); PSD-95, postsynaptic density protein 95.

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hypertension according to studies in knockout mice [7–9]. While expression of the α_1/β_1 form is predominant in the cardiovascular system, recent findings point to an unwanted sympathostimulatory action mediated by the α_1/β_1 form in the medulla oblongata [10]. This puts the α_2/β_1 heterodimer in the spotlight as a specific drug target.

Zabel et al. have first shown that co-expression of α_1 and β_1 subunits in overexpression systems leads to the formation of α_1 and β_1 homodimers in addition to heterodimers [11]. We have confirmed that homodimer formation of α_1, α_2 and β_1 subunits occurs after overexpression of the respective subunit in Sf9 cells [3]. In addition, we have shown that a separate pool of α and β_1 subunits exists in brain tissue in vivo [12]. These non-heterodimerizing subunits may exist as monomers, may bind to chaperones or may form homomers. It has even been suggested that non-heterodimerizing subunits have a separate function from cGMP signaling: The α_1 subunit is thought to act as a mediator of the procarcinogenic effect of androgens [13–15]. The β_1 subunit has been implicated in chromatin condensation and cell cycle progression [16]. To isolate the effects of α_1/β_1 and α_2/β_1 heterodimeric enzyme complexes from effects of non-heterodimerizing α and β_1 subunits, we cloned and characterized conjoined NOsGCs [3,17]. In these constructs the β_1 and α subunits are joined by a fluorescent protein or a linker sequence. On over-expression of conjoined NOsGCs, no homomeric α and β subunits can be formed. Instead, intramolecular dimerization between the α and β parts of the protein leads to the formation of enzymes with properties indistinguishable from the respective heterodimers [3].

In the present paper we analyzed the subcellular localization of both enzyme isoforms. With the use of a conjoined fluorescent β_1YFPα_2 construct, we detected a localization at cell-cell contacts upon overexpression in human embryonic kidney cells (HEK293). This was confirmed by co-expression of α_2 and β_1 subunits, while the isolated α_2 subunits showed no such localization. We conclude that heterodimerization is a prerequisite for further protein-protein interactions that direct the α_2/β_1 form to specific subcellular sites important for signaling. Further experiments in HEK293 cells indicate that the adapter protein Lin7a directs the α_2/β_1 heterodimer to nectin based cell-cell contacts. The interaction of Lin7a with the α_2/β_1 heterodimer in vivo was confirmed by co-precipitation from mouse brain synaptosomes.

2. Materials and methods

2.1. Materials

Unless stated otherwise, chemicals in the highest grade of purity were obtained from Sigma-Aldrich (Steinheim, Germany). Cell culture medium was received from Fisher Scientific (Waltham, USA). All restriction enzymes were obtained from New England Biolabs (Ipswich, USA), pECFP and pEYFP vectors (cyan fluorescent protein, yellow fluorescent protein) were from Clontech (Mountain View, USA). Mutageneses were performed with QuikChange Lightning Mutagenesis Kit from Agilent (Santa Clara, USA). The oligonucleotides were obtained from Biomers (Ulm, Germany).

2.2. Tissue and cell culture

HEK293 cells were obtained from the DSMZ (German Collection of Microorganisms and Cell Culture, Braunschweig). Cells were cultivated in Dulbecco's Modified Eagle Medium-High Glucose (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C with 5% CO_2. For microscopic analysis, the cells were seeded in imaging plates with a special glass bottom (SensoPlate, Greiner bio-one, Kremsmünster, Austria). Polyethylenimine was used for the transfection with cDNA encoding for fluorescently labelled proteins. The incubation time was at least 48 h. Passages 10–20 were used for the experiments.

2.3. RNAi experiments

Two chemically synthesised siRNAs Silencer Select® s16834 and s16835 from Thermo Fisher Scientific (Waltham, USA) were used to knock down endogenous expression of Lin7a in HEK293 cells. The negative control was transfected with two scrambled Silencer select® siRNAs. RNAi transfections were done in a 35 mm Dish (μ-Dish, ibiTreat, Ibidi, Munich, Germany). 24 h after reverse transfection with siRNA using Lipofectamin® RNAIMAX (Thermo Fisher Scientific) cells were transfected with cDNA encoding for fusion protein β_1YFPα_2 using Lipofectamin® LTX (Thermo Fisher Scientific). Cells were analyzed by confocal laser scanning microscopy (CLSM) 48 h after the second transfection and the same samples were analyzed by SDS-PAGE and western blotting immediately after microscopy.

2.4. Tissue specimen

Female NMRI (Naval Medical Research Institute) mice aged between 12 and 22 weeks were killed by decapitation. The brain was immediately frozen and stored at −20 °C until protein preparation. Animals were from Charles River and research was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

2.5. Brain preparation

Mouse brain was homogenized using a glass pestle of a Potter-Elvehjem homogenizer in four volumes of buffer A (0.32 M sucrose, 1 mM EDTA, 2 mM 1.4-dithiothreitol and 10 mM HEPES, pH 7.4, containing complete™ protease inhibitor cocktail from Roche (Basel, Switzerland)). This homogenate was diluted 1:5 in buffer A. After low speed centrifugation (750g, 10 min) the pellet was discarded. The resulting supernatant was centrifuged at 15,000g for 10 min. Following this centrifugation, the second supernatant was kept as cytosolic fraction. The pellet was resuspended in buffer A and solubilised with Triton X-100 at a final concentration of 0.5%. After 10 min incubation on ice, the Triton X-100 extract was centrifuged (15,000g, 20 min). The supernatant was used as crude synaptosomal/membrane fraction for co-immunoprecipitation [6]. Triton X-100 was also added to the cytosolic fraction to achieve equal treatment. The protein concentrations were determined by the method of Bradford using bovine serum albumin as standard.

2.6. NOsGC activity assay

NOsGC activity was quantified as described in Busker et al. 2014 [18]. The activity of the obtained HEK293 cell homogenate samples (0.3–0.5 mg total protein/assay tube) was determined by incubation for 10 min at 37 °C in the presence of 50 mM triethanolamine/HCl buffer pH 7.4, containing 3 mM dithiotreitol, 1 mM 3-isobutyl-1-methylxanthine, 1 mM cGMP, 5 mM creatine phosphate, 10 units/tube creatine phosphokinase, 0.5 mM [α-^{32}P] GTP and 3 mM $MgCl_2$. The reaction was stopped after 10 min by adding 500 μl of 125 mM zinc acetate and 500 μl of 120 mM sodium carbonate leading to co-precipitation of zinc carbonate and 5′-nucleotides. For the dose–response curves, 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO) was used in concentrations ranging from 1 nM to 1 mM.