Nitric Oxide 77 (2018) 44–52

Contents lists available at ScienceDirect

Nitric Oxide

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

Measurement of cGMP-generating and -degrading activities and cGMP levels in cells and tissues: Focus on FRET-based cGMP indicators

Michael Russwurm*, Doris Koesling

Pharmakologie und Toxikologie, Medizinische Fakultät, Ruhr-Universität Bochum, Bochum, Germany

ARTICLE INFO

Keywords:
Fluorescence resonance energy transfer
Cyclic guanosine monophosphate
Nitric oxide
Guanylyl cyclase
Phosphodiesterase

ABSTRACT

The intracellular messenger molecule cGMP has an established function in the regulation of numerous physiological events. Yet for the identification of further biological cGMP-mediated functions, precise information whether a cGMP response exists in a certain cell type or tissue is mandatory.

In this review, the techniques to measure cGMP i.e. cGMP-formation, -degradation or levels are outlined and discussed. As a superior method to measure cGMP, the article focusses on FRET-based cGMP indicators, describes the different cGMP indicators and discusses their advantages and drawbacks. Finally, the successful applications of these cGMP indicators to measure cGMP responses in cells and tissues are outlined and summarized.

Hopefully, with the availability of the FRET-based cGMP indicators, the knowledge about the cGMP responses in special cells or tissues is going to increase thereby allowing to assess further cGMP-mediated functional responses and possibly to address their pathophysiology with the available guanylyl cyclase activators, stimulators and PDE inhibitors.

1. Introduction

cGMP plays an important role as a signaling molecule and is involved in a pleiotropy of physiological responses. In analogy to cAMP, cGMP is formed by guanylyl cyclase and is degraded by phosphodiesterases (PDEs) and many of the cyclic nucleotide effects are transduced by the respective protein kinases. Compared to cAMP, cGMP levels are lower than cAMP levels in many cells and tissues. In addition to smooth muscle relaxation, alteration of synaptic transmission, inhibition of platelet aggregation and of salt and water reabsorption (for review see Ref. [1]), cGMP has been proposed to affect cell growth and survival although with conflicting results (see e.g. Ref. [2] vs. [3]). cGMP has been proposed to occur in most tissues, yet, it is unclear whether cGMP occurs in most of the cells types in a certain tissue or whether the cGMP content is simply due to the vascularization of the tissue. Consequently, the occurrence of the cGMP signaling cascade in most cells is unclear as are the functional responses elicited by cGMP. Therefore, the determination whether relevant cGMP levels occur in certain cell types must be considered as a key step in the identification of cGMP-induced events.

In the cellular context, cGMP is formed by the membrane-spanning natriuretic peptide receptor-coupled-guanylyl cyclases and by the guanylyl cyclases stimulated by nitric oxide. Two major receptor-coupled GCs (GC-A, GC-B) exist and are stimulated by ANP and BNP in the case of GC-A and CNP for GC-B and also two NO-sensitive GCs (NO-GC1, NO-GC2) with indistinguishable regulatory and catalytic properties have been shown to occur. As one of the NO-GC isoforms, NO-GC2, is able to interact with the scaffolding protein PSD-95, membrane association may reflect the special properties of this NO-GC isoform.

Whereas the early research focused on the isolation and characterization of NO-GCs, now the biological effects induced by cGMP are of major interest. Here, we will describe and discuss techniques used to measure cGMP formation, cGMP degradation, and cGMP levels in cells and tissues.

2. Measurements of cGMP-forming activity

cGMP-forming activity of cell homogenate can be determined by the conversion of GTP to cGMP as described in detail by Schultz and Böhme [4]; a shorter and better available description has been published in Methods in Enzymology [5]. In this assay, radioactively labelled [α-^{32}P] GTP used as a substrate gives rise to [^{32}P] cGMP, which can be counted after removal of the substrate. Unfortunately, the assay is not very sensitive and the amount of homogenate that can be applied is rather low. Higher amounts of homogenate result in the inhibition of the cGMP-forming activity and an underestimation of the cGMP formation in a given tissue. Therefore, the amount of homogenate that can

* Corresponding author. Pharmakologie und Toxikologie, Med. Fak MA N1, Ruhr-Universität Bochum, 44780, Bochum, Germany.
E-mail address: michael.russwurm@ruhr-uni-bochum.de (M. Russwurm).

https://doi.org/10.1016/j.niox.2018.04.006
Received 26 February 2018; Received in revised form 17 April 2018; Accepted 18 April 2018
Available online 21 April 2018
1089-8603/

be applied has to be carefully experimentally determined. Due to these limitations, one can only detect cGMP formation in the homogenate of cells with very high GC content (> 1 nmol/mg/min) such as platelets, lung, smooth muscle cells, brain or overexpressing cells.

The sensitivity of the assay can be improved by the detection of the formed cGMP in radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA) which are commercially available. In that case, the radioactively labelled GTP is not required and ordinary GTP is used as substrate. Careful controls are required to exclude cross-reactivity of the substrate with the cGMP antibody; we found some batches of commercial GTP to contain considerable amounts of cGMP. With this assay, roughly 10-times lower rates of NO-stimulated cGMP forming activities are measurable.

As outlined above, due to an impairment of the assay conditions, only a limited amount of homogenate can be applied in the assay measuring cGMP-forming activity. The problem can be circumvented by the preparation of a NO-GC enriched fraction by anion exchange chromatography, as in the first step of NO-GC purification [5]. Here, the homogenate is incubated with Q Sepharose with low salt either in batch mode or by chromatography. After washing away unbound proteins, the bound protein is eluted with a buffer containing 250 mM NaCl in appropriate fractions. Subsequently, NO-stimulated cGMP formation is determined in the fractions. Usually, cGMP-forming activity and the protein content co-elute.

3. Measurements of phosphodiesterase activity

A classical assay of PDE activities has been described by Thompson and Appleman [6] and recently a detailed methodological paper containing a comprehensive review of the methods available has been published by Rybalkin et al. [7]. We are routinely using a different method shortly described in Jäger et al. [8]; however this method requires synthesis of [^{32}-P] cAMP and [^{32}-P] cGMP from commercially available [α-^{32}P] ATP and [α-^{32}P] GTP, respectively. This can be achieved using purified NO-GC (which also produces cAMP with ~10-fold lower activity than cGMP) in analogy to the guanylyl cyclase assay described above. However, as commercially available NO-GC is expensive, this method might be attractive only to those purifying NO-GC.

As the PDE activity mainly depends on the substrate concentration used, the resulting activity does not necessarily reflect the activity of the PDEs within the cell. E.g. smooth muscle cells contain the cGMP-hydrolyzing PDEs 1, 3 and 5 [9]. PDE3 (kM ~ 20 nM [10]) will play a larger role at nanomolar substrate concentrations. PDE5 (kM 3–6 μM [10]) which is allosterically activated by cGMP in the micromolar range [11] will predominate in PDE activity assays with micromolar cGMP substrate concentrations unless calcium/Calmodulin is included leading to PDE1 activation (kM_{cGMP} ~ 1–3 μM). Thus, these measurements are used to identify the PDEs present in a certain cell or tissue with the help of PDE inhibitors or to study the regulation of PDEs.

4. Determination of cGMP levels by antibody-based methods

As outlined above, the level of cGMP in a certain tissue depends not only on the formation of cGMP but is strongly regulated by its degradation through PDEs. Therefore, the determination of cGMP levels the in the absence and presence of PDE inhibitors provides information about the relative contribution of the GCs and PDEs in a given tissue. Yet, to be able to judge the interplay of cGMP formation and degradation, incubation with the cGMP increasing reagents (nitric oxide, natriuretic peptides) and or PDE inhibitors has to be performed with intact cells or tissues. Afterwards, the cells are disrupted and cGMP is typically detected by antibody-based methods. A cGMP radioimmunoassay has been described by Steiner et al. [12], and recently a detailed protocol has been published [13]. However, the cGMP radioimmunoassay requires generation of cGMP antibodies and frequent radioactive labelling of cGMP, which is not commercially available any more. More conveniently, ready to use-cGMP ELISAs are commercially available but expensive.

Table 1

Tissue	cGMP (pmol/mg protein)	Measurement condition	Ref.
rat aorta	2	unstimulated	[73]
	66	1 μM SNP	
rabbit aorta	0.5	unstimulated	[76]
	1.5	10 nM ANP	
bovine arteria & veins	0.45[a]	unstimulated	[77]
	3[a]	100 μM bradykinin	
mouse aorta	10	unstimulated	[74]
	100	100 μM GSNO	
rat aorta	180	100 μM GSNO	[78]
rat bronchus	25	100 μM GSNO	[78]
human platelets	175[b]	300 μM GSNO	[79]
	1500[b]	300 μM GSNO, 100 μM sildenafil, 100 μM EHNA	
rat platelets	300	50 nM NO (clamped)	[80]
	1000	50 nM NO (clamped), 100 μM sildenafil	
human platelets	6	10 mM SNP, 100 nM sildenafil, 3 min	[81]
rat brain stem slices	0.035	unstimulated	[82]
	0.34	0.1 μM BAY41-2272	
	0.135	10 μM GTN	
	1.7	0.1 μM BAY41-2272, 10 μM GTN	
rat hippocampal slices	2	300 μM EHNA	[83]
	15	300 μM EHNA, 100 μM NMDA	
	30	300 μM EHNA, 10 μM DEA-NO	
mouse visual cortex	7	1 mM IBMX	[84]
	40	1 mM IBMX, 10 μM DEA-NO	
mouse hippocampal slices	10	300 μM IBMX	[85]
	40	300 μM IBMX + 10 μM DEA-NO	
	20	300 μM IBMX, 300 μM NMDA	
rat hippocampal slices	1000	10 μM DEA-NO, 10 μM BAY41-2272, 1 μM BAY60-7550	[86]
mouse hippocampal slices	4	1 μM BAY60-7550	[87]
	20	1 μM BAY60-7550, 100 μM NMDA	
mouse kidney slices	0.5	unstimulated	[88]
	10	100 μM DEA-NO	

[a] Calculated from pmol/g wet weight using 1 mg protein/15 mg wet weight as rule of thumb.

[b] Calculated from pmol/10^9 platelets using 2 mg protein/10^9 platelets as rule of thumb.

Typical cGMP contents of different tissues or cells, measured using radioimmunoassays or ELISAs are given in Table 1.

Because measurement of cGMP in tissues by radioimmunoassays or ELISAs requires homogenization, only mean cGMP values averaged about all cells are obtained, i.e. in complex tissues, information about the cells responsible is lost. To overcome this limitation, an immunohistochemical approach using cGMP antibodies has been developed by de Vente et al. [14]. However, spatial information comes at the cost of quantitative resolution, which is relatively low with this method [15].

5. cGMP measurements with genetically encoded FRET indicators

To overcome the limitations of antibody-based cGMP measurements such as low temporal and local resolution, a first fluorescence resonance energy transfer (FRET)-based cGMP sensor was developed shortly after publication of a genetically encoded cAMP sensor [16] by the Dostmann group [17]. The term 'genetically encoded' is used to distinguish this type of sensors for second messengers from small organic molecules used e.g. for the measurement of intracellular Ca^{2+} [18] and earlier cAMP sensors consisting of the catalytic and regulatory subunits of cAMP-dependent protein kinase labelled *in vitro* with different fluorescent dyes [19].