Negative feedback in NO/cGMP signalling

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Abstract

Most of the effects of the signalling molecule nitric oxide (NO) are mediated by the stimulation of the NO-sensitive GC (guanylate cyclase) and the subsequent increase in cGMP formation. The enzyme contains a prosthetic haem group, which mediates NO stimulation. In addition to the physiological activator NO, NO-sensitizers like the substance YC-1 sensitize the enzyme towards NO and may therefore have important pharmacological implications. Two isoforms of NO-sensitive GC have been identified to date that share regulatory properties, but differ in the subcellular localization. The more ubiquitously expressed α1β1 heterodimer and the α2β1 isoform are mainly expressed in brain. In intact cells, NO-induced cGMP signalling not only depends on cGMP formation, but is also critically determined by the activity of the enzymes responsible for cGMP degradation, e.g. PDE5 (phosphodiesterase 5). Recently, direct activation of PDE5 by cGMP was demonstrated, limiting the cGMP increase and thus functioning as a negative feedback. As the cGMP-induced PDE5 activation turned out to be sustained, in the range of hours, it is probably responsible for the NO-induced desensitization observed within NO/cGMP signalling.

Introduction

Nitric oxide (NO) formed by the NO synthases throughout the body plays an important role in the cardiovascular system [1] and is involved in the modulation of synaptic transmission [2]. Most of the effects of the signalling molecule NO are mediated by the activation of the NO-sensitive GC (guanylate cyclase) [3], the enzyme which by increasing cGMP transduces the NO signal to the cGMP-regulated protein kinases, cGMP-activated PDEs (phosphodiesterases) and cGMP-gated ion channels. cGMP is degraded by the PDEs, which thereby terminate the cGMP-signal [4].

Enzymes involved in the NO/cGMP signalling cascade are important drug targets. The so-called NO donors used in the therapy of coronary heart disease exert their action via the release of NO and the subsequent GC activation. The newly developed NO-sensitizers also work on the level of GC, but instead of substituting for NO, they sensitize the enzyme towards NO and thereby enhance the effects of endogenous NO. In addition to stimulation of cGMP synthesis, inhibition of cGMP degradation has been shown to be very effective as demonstrated by the successful therapeutic application of the PDE5 inhibitors like sildenafil. In the following report, we will summarize some recent findings about NO-sensitive GC and a feedback loop within NO/cGMP signalling mediated by cGMP-induced activation of PDE5.

Two isoforms of NO-sensitive GC

GC is a haem-containing enzyme and consists of two different subunits, α and β (Figure 1). Two isoforms have been shown to exist containing the β1 and either the α1 or the α2 subunits [5]. The α1 and α2 subunits show pronounced homology in the C-terminal catalytic domains, which are also conserved in the membrane-bound GC and in the cytosolic regions of the adenylate cyclases. However, the N-terminal regions differ considerably. The physiological impact of another subunit, β2, identified on the level of sequence homology is unclear. So far, this subunit has not been expressed as a catalytically active enzyme and the mRNA content identified in the quantitative PCR was very low at least in mice [6].

Dimerization of the subunits has been shown to be a prerequisite for enzyme activity. Whereas for a long time, the

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Abbreviations used: GC, guanylate cyclase; HEK-293 cells, human embryonic kidney 293 cells; PDE, phosphodiesterase.

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regions preceding the catalytic domains were considered to be sufficient for dimerization [7], in a recent report the additional presence of another region has been shown to be mandatory for a stable subunit interaction [8]. This region was located in the N-terminal parts of the subunits. Without this N-terminal interaction-site, no quantitative heterodimerization between α and β occurs, but rather homodimerization of the β₁ subunit. As has been published by others as well, the β₁ subunit has a strong tendency to form homodimers, which probably is the reason for the robust expression of the β₁ subunit observed in various expression systems. The N-terminal region of the α₁ subunit (amino acids 61–128) and the central domain (amino acids 367–462) are required to outcompete homodimerization of the β₁ subunit. If one of the regions is not present and dimerization thus impaired, the α₁ subunit is degraded resulting in a low yield of truncated α₁ subunits in expression systems. These results, i.e. the different stability of GC subunits, should be kept in mind in further experiments with truncated GC subunits.

Despite the differences in the primary structures of the N-terminal haem binding regions of the α₁ and α₂ subunits, no functional differences could be detected between the isoforms with regard to haem co-ordination, NO-sensitivity and catalytic activity. However, the α₂ subunit contains a C-terminal peptide which is able to interact with PDZ domains and the α₂β₁ isoform has been shown to be associated with the post-synaptic adapter protein PSD-95 [9].

The occurrence of the GC isoforms has been studied with quantitative PCR and Western blot-analysis. The highest abundance for the α₂ subunit was detected in brain; together with the reported PSD-95 interaction, a special role of the α₂β₁ in synaptic transmission appears feasible [6]. On the other hand, the α₁β₁ isoform is not the only NO-sensitive GC in brain as the α₁β₁ heterodimer is expressed in similar amounts. In all other tissues tested, the α₁β₁ is the major isoform with the highest amount found in lung and aorta and other highly vascularized tissues. The α₂β₁ isoform could be detected in all of the organs studied although with a very low abundance.

**Activation of NO-sensitive GC**

GC contains a prosthetic haem group that is required for NO stimulation. The haem has an absorption maximum at 431 nm indicating a five-co-ordinated haem with the amino acid histidine as the axial ligand [10]. After different intermediate steps proposed recently, NO binding to the sixth co-ordination position of the haem iron finally results in a five-co-ordinated haem, in which the bond to the proximal histidine is broken. The activated enzyme exhibits a 200-fold increased catalytic rate [11]. This NO-bound five-co-ordinated haem shows an absorption maximum at 399 nm. Because of the pronounced change in absorbance, binding of the ligand, NO, to its receptor, GC, can be monitored spectrophotometrically; NO binding and activation have been assumed to always coincide.

However, recent results indicate the presence of two spectrophotometrically indistinguishable NO-bound states of the GC, which differ in catalytic activity [12]. NO binding in the presence of the substrate GTP or the reaction products (cGMP and PPi) yields the highly activated GC species, whereas NO binding in the absence of the substrate as usually performed in spectral studies leads to the formation of the non-activated NO-bound GC state. Under physiological conditions, GTP should always be present in the GC environment. Therefore the non-activated NO-bound state probably represents an artifact due to the artificial absence of the substrate. As the catalytic rate is not the only difference between the two NO-bound states, GTP should be included in further spectral studies of the enzyme to ensure that the naturally occurring NO-bound activated state is studied.

**The NO/cGMP response**

In intact cells, the cGMP formed by the GCs is hydrolysed by PDEs. Therefore the amplitude and the duration of the cGMP response critically depend on the amount and regulation of the cGMP-synthesizing and -degrading enzymes in a certain tissue [4].

Platelets contain high levels of NO-sensitive GC and PDE5 as the major enzyme responsible for cGMP degradation. The NO-induced cGMP response measured in platelets is characterized by a fast increase in cGMP within 5–10 s followed by a rapid decline of cGMP, which is almost back to resting levels after 30–40 s (Figure 2) [13,14]. Furthermore, NO causes a desensitization of the cGMP response, which can be seen for as long as 1 h after the NO exposure.

Recently, direct activation of PDE5 by cGMP has been demonstrated. PDE5 has a homodimeric structure and,
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**Figure 2** | Negative feedback in NO/cGMP signalling

The NO-induced cGMP response in platelets is characterized by the transient shape and the occurrence of desensitization upon NO restimulation. Within the cGMP-response, cGMP leads to the activation of PDE5 explaining the rapid decrease of intracellular cGMP and the desensitization. Parallel to the activation, PDE5 is phosphorylated by cGMP-dependent protein kinase, increasing the sensitivity of PDE5 for cGMP. This feedback mechanism may regulate the sensitivity of the NO/cGMP signalling cascade.

besides the catalytic cGMP-hydrolysing sites, contains two N-terminal regulatory domains, the so-called GAF domains initially found in cGMP-PDEs, adenylyl cyclases and *Escherichia coli* FhlA (for a review, see [15]). cGMP was shown to induce PDE5 activation [16] by binding to the GAF-A domain [17]. The cGMP-induced PDE5 activation is only observed in fresh enzyme preparations, after storage enzyme activity increases and cGMP-responsiveness is lost [17].

The NO-induced cGMP-mediated allosteric activation of PDE5 most likely is responsible for the transient shape of the cGMP response in platelets and is also sufficient to explain the NO-induced desensitization, i.e. the reduced NO-responsiveness upon NO restimulation. In cells, parallel to activation, PDE5 is phosphorylated by the cGMP-dependent protein kinase in its N-terminal region [18,19]. Although there have been reports that phosphorylation increases the affinity towards cGMP [20], the precise role of PDE5 phosphorylation has not been demonstrated.

In order to elucidate the functional role of phosphorylation, the cGMP-dependent activation of PDE5 was studied in cytosolic preparations of HEK-293 cells (human embryonic kidney 293 cells) expressing PDE5 [21]. Phosphorylated and non-phosphorylated PDE5 were incubated with increasing cGMP concentrations (5 min), and subsequently, activity was determined at a low cGMP concentration of 0.1 µM. As can be seen in Figure 3(A), cGMP concentration-dependently led to an approx. 11-fold activation of PDE5 under these experimental conditions. However, the concentration-responses for cGMP activation of phosphorylated and non-phosphorylated PDE5 were almost indistinguishable. Next, the time course of the PDE5 deactivation...

**Figure 3** | The effect of phosphorylation on activation and deactivation of PDE5

(A) Activation of phosphorylated and non-phosphorylated PDE5 by preincubation with increasing cGMP concentrations. (B) Deactivation of phosphorylated and non-phosphorylated PDE5. PDE5 (phospho and non-phospho) was activated by preincubation with 30 µM cGMP. Then, samples were diluted to yield a cGMP concentration of <10 nM and were further incubated. At the time-points indicated, PDE5 activity was determined.
was studied to find out how long the activation persists and to elucidate a possible effect of phosphorylation. Phosphorylated and non-phosphorylated PDE5 were activated with 30 μM cGMP (5 min). Subsequently, the samples were diluted 1000-fold (residual cGMP <10 nM) and the time course of deactivation was monitored by measuring PDE5 activity at the indicated time points. As can be seen in Figure 3(B), phosphorylation of PDE5 had a profound impact on the rate of deactivation at 10 nM cGMP. While activation of non-phospho-PDE5 was completely reversed after 16 min, phospho-PDE5 was still substantially activated (∼60%).

Under these experimental conditions, the estimated half-lives for activated non-phospho- and phospho-PDE5 were 3 and 20 min respectively. Clearly, phosphorylation prolonged the activation of PDE5 most likely by increasing the affinity towards cGMP. Indeed, a higher ambient cGMP concentration during deactivation was able to compensate for the effect of phosphorylation as the deactivation of non-phosphorylated PDE5 at a cGMP concentration of 50 nM was as slow as the deactivation of phospho-PDE5 seen at 10 nM cGMP. Summarizing, phosphorylation of PDE5 prolongs activation but even without phosphorylation, PDE5 activation is very sustained and therefore might well account for the NO-induced desensitization observed in platelets.

However, the question remained whether factors other than GC and PDE5 are required for the shape and desensitization of the cGMP response. To answer this, the signalling cascade was reconstituted in HEK-293 cells. The NO-induced cGMP response determined in HEK-293 cells, stably transfected with both subunits of NO-sensitive GC and PDE5, showed the characteristic transient shape of cGMP reminiscent of the one observed in platelets. Also, activation of PDE5 occurred as measured in the cytosol of NO-incubated cells. Moreover, similar to platelets, NO induced a long-lasting desensitization of the cGMP response in HEK-GC/PDE5 cells.

In summary, the characteristic shape and the desensitization of the cGMP response observed in platelets also occurs in cells solely transfected with NO-sensitive GC and PDE5. We conclude that these features are inherent to these enzymes and therefore are likely to occur in all GC- and PDE5-expressing cells. Forthcoming experiments have to clarify the functional relevance of the desensitization occurring within NO/cGMP signalling.

References

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