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NO-SENSITIVE GUANYLYL CYCLASE AND NO-INDUCED FEEDBACK INHIBITION IN cGMP SIGNALING

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TABLE OF CONTENTS

1. ABSTRACT

Most effects of the signaling molecule nitric oxide (NO) are mediated by the stimulation of NO-sensitive guanylyl cyclase (GC) and the subsequent intracellular increase in cGMP. Two isoforms of NO-sensitive GC have been identified to date that share regulatory properties but differ in their subcellular localization; the more ubiquitously expressed alpha1beta1 heterodimer, and the alpha2beta1 isoform mainly expressed in brain. New activators of NO-sensitive GC have been identified which may have beneficial pharmacological effects in cardiovascular diseases.

In intact cells, NO-induced cGMP signaling not only depends on cGMP formation but is also critically determined by the activity of the enzyme responsible for cGMP degradation, e.g. phosphodiesterase 5 (PDE5). Sustained activation of PDE5 by cGMP has been identified as the mechanism responsible for the recently observed feedback inhibition within NO/cGMP signaling. Moreover, tuning of PDE5 activity may also represent a regulatory link to mediate cross talk between NO-induced and natriuretic peptide-induced cGMP signaling in general.

2. INTRODUCTION

Cyclic GMP, like cAMP, has important functions as intracellular signaling molecule in the regulation of various cellular events. Although many of the biological functions of cGMP still remain to be discovered, it is an established messenger molecule involved in smooth muscle relaxation, platelet aggregation and neurotransmission. Cyclic GMP exerts its effects through cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases and cGMP-regulated ion channels. Two major types of cGMP-forming enzymes have been identified: the membrane-spanning peptide-regulated guanylyl cyclases and the NO-sensitive guanylyl cyclases (NO-sensitive GCs). Cyclic GMP is degraded by phosphodiesterases with PDE5 being the major cGMP degrading enzyme in many tissues. Accordingly, the intracellular cGMP concentration is a result of the respective activities of NO-sensitive GC and PDE.

NO has been shown to induce a transient spike-like cGMP signal in many cell types indicative of a dynamic regulation of the underlying cGMP-forming and -degrading activities. Within this cGMP response, binding of NO to the regulatory heme moiety of NO-sensitive GC can been seen as on-switch of cGMP synthesis. Direct cGMP-mediated activation of PDE5 has been demonstrated to be responsible for the decline of the intracellular cGMP concentration. Thus, the signaling molecule cGMP not only mediates downstream effects but also activates its own degradation by a regulatory feedback mechanism.

This review summarizes the most important properties of the cGMP-forming enzyme NO-sensitive GC and describes NO-dependent and NO-independent mechanisms regulating enzyme activity; furthermore, it will outline the recently identified NO-induced feedback inhibition within cGMP signaling that allows a given cell or tissue to adapt to the NO available.

3. STRUCTURE AND REGULATION OF NO-SENSITIVE GC

3.1. Subunit composition and tissue distribution

NO-sensitive GC is a heterodimeric enzyme consisting of two subunits, alpha and beta (Figure 1).

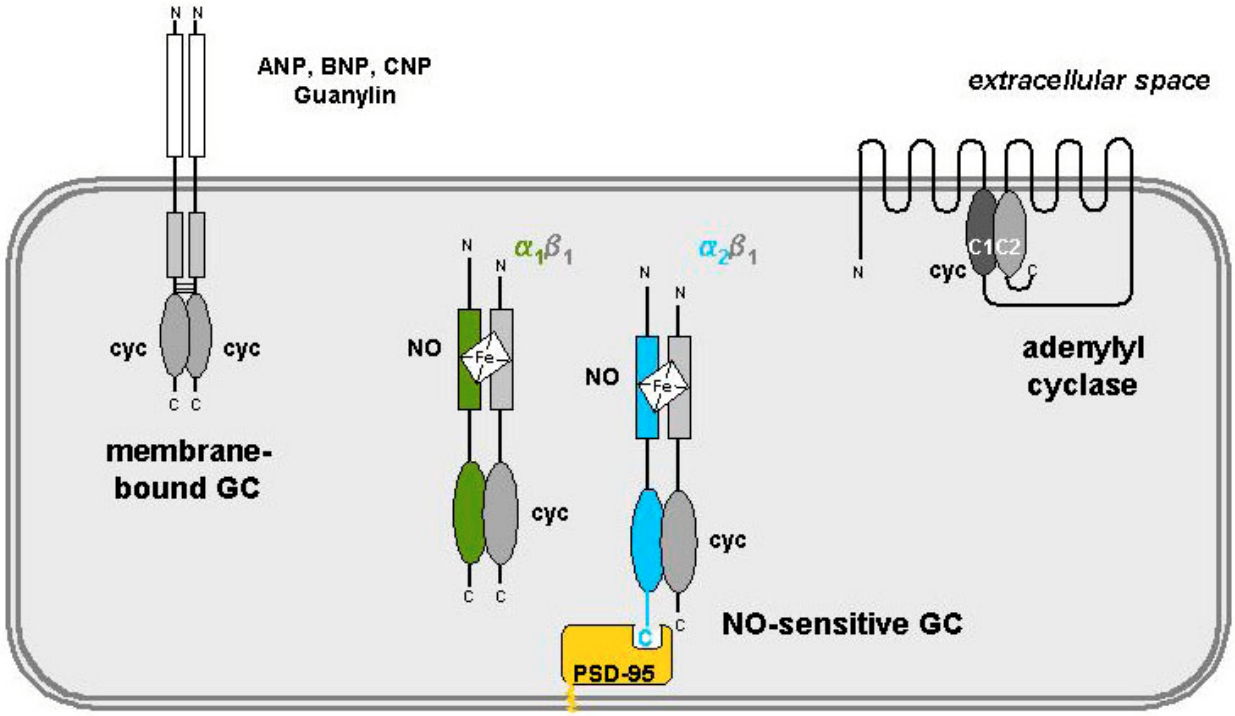

Figure 1. Schematic representation of the overall structures of guanylyl cyclases and adenylyl cyclases. Shown are the known heterodimers of NO-sensitive guanylyl cyclase (alpha1/beta1, alpha2/beta1), a representative membrane-bound guanylyl cyclase with different ligands at the respective receptor domain, and a representative adenylyl cyclase. The catalytic domains (labelled with cyc) are symbolised by ellipses. The heme group with its central iron is bound to the N-terminal domains of NO-sensitive GC. The alpha2 subunit of NO-sensitive GC is depicted in blue. By binding to the very C-terminal amino acid residues of this subunit, the post-synaptic density protein PSD95 localizes the alpha2/beta1 isoform to the cell membrane (for further information see text). ANP, natriuretic peptide type A; BNP, natriuretic peptide type B; CNP, natriuretic peptide type C; cyc, catalytic domain of guanylyl cyclases and adenylyl cyclases; PSD95, post-synaptic density protein 95.

Subsequent to the purification of the enzyme from lung tissue (1-4) both subunits were cloned, sequenced and termed alpha1 and beta1. Homology screening allowed for the identification of two additional subunits, which by comparison to the known subunits were termed alpha2 and beta2 (5, 6). Formation of a catalytically active and highly NO-sensitive enzyme (alpha2/beta1) was only shown after coexpression of the alpha2 subunit with the beta1 subunit in COS cells (5) whereas coexpression with the alpha1 subunit did not result in a functional guanylyl cyclase. The alpha2 subunit has indeed been shown to occur in native tissue. An alpha2/beta1 heterodimer was also purified from porcine lung (Russwurm *et al.*, personal communication). Proof of physiological importance of the beta2 subunit is still lacking. In most cases, the beta2 subunit did not yield a GC sensitive to NO when coexpressed with either alpha1, alpha2 or beta1 subunits. Data on the quantitative tissue distribution (see below) suggests the beta2 subunit not to exist on the protein level. This assumption is underlined by the identification of a frameshift in the human gene of the beta2 subunit that is incompatible with its occurrence on the protein level in humans (7).

Two functional domains can be located on each subunit of NO-sensitive GC, the catalytic core in the C-terminal domain and the regulatory heme-binding domain in the N-terminal region which mediates NO stimulation (see below). The catalytic domains are highly conserved among the subunits of NO-sensitive GC and show homologies to the respective regions in the peptide receptor guanylyl cyclases and the adenylyl cyclases. The catalytic domain of adenylyl cyclase has been crystallized and it was shown that the two catalytic domains are organized in an antiparallel orientation (8, 9). From these data, the structure of the respective domain on NO-sensitive guanylyl cyclase has been modeled; crystallization of NO-sensitive GC, so far not accomplished, will expose the structural similarities and differences between the two enzymes. Binding of the prosthetic heme moiety was shown to require a conserved histidine residue on the beta subunit. Exchange of this residue with phenylalanine resulted in a non-NO-sensitive mutant which retained basal activity (10, 11). In a recent report, two additional amino acid residues, tyrosine-135 and arginine-139 on the beta subunit, were shown to be involved in heme binding. Interaction of these two residues appears to occur with the propionic side chains of the heme moiety (12).

The regions responsible for dimerisation of the two subunits of NO-sensitive GC have not yet been unequivocally determined. Mainly based on studies on the peptide receptor guanylyl cyclases (13), dimerisation has so far been attributed to occur in the central parts of the subunits. Further support came from truncated mutants,

where deletion of large N-terminal parts of the alpha1 subunit did not prevent dimerization nor abolished basal enzymatic activity (14). However, recent data indicate that the simple model of one central 'dimerisation domain' is not valid. Rather, two discontinuous sites appear to be responsible for dimerisation of the subunits (15).

3.2. Different subcellular targeting of the alpha1/beta1 and alpha2/beta1 isoforms

For a long time, the physiological significance of two isoforms, alpha1/beta1 and alpha2/beta1, was unclear. Despite divergent amino acid sequences of the N-terminal parts of the alpha subunits, both isoforms share similar features with respect to enzymatic activity and regulation by NO. In 2001, the alpha2/beta1 isoform was demonstrated to be associated with the PDZ-containing postsynaptic density protein-95 (PSD-95) via the very C-terminal amino acids of the alpha2 subunit (—FLRETSL; Figure 1). As a result of the interaction with PSD-95 the alpha2/beta1 isoform is associated with synaptosomal membranes in brain (16); therefore, the terms 'soluble' or 'cytosolic' guanylyl cyclase used so far are misleading, instead we use the term 'NO-sensitive guanylyl cyclase' in this review. Location of NO-sensitive GC is not restricted to the postsynaptic side. Immunohistochemistry of the CA1 region of the hippocampus revealed a postsynaptic localization of the neuronal NO synthase and a presynaptic signal with antibodies against the beta1 subunit (17). Thus, one might speculate about a vis-à-vis location of GC and NO synthase with nitric oxide as retrograde messenger. Further data on the relative distribution of the GC isoforms argues in favor of the alpha2/beta1 as the neuronal isoform of NO-sensitive GC (see below).

Translocation of the alpha1/beta1 isoform to the plasma membrane in response to elevated calcium concentrations has been reported. This membrane association which has been attributed to an interaction with heat shock protein 90 (18) resulted in an increase in NO sensitivity of the enzyme in heart tissue (19). Yet, this sensitization towards NO has been disputed recently in cytosolic and membrane fractions of cerebellar tissue and platelets (20).

3.3. Tissue distribution

The tissue distribution of NO-sensitive GC has been studied on RNA and protein levels. Northern blot analysis revealed a broad distribution of both the alpha1 and beta1 subunits which is in accordance with the notion of the alpha1/beta1 heterodimer being the predominant isoform. The alpha2 subunit was only detected in brain, placenta and uterus (21). In rat brain, a widespread distribution of the alpha1, alpha2 and beta1 subunits was demonstrated by RT-PCR and *in situ* hybridization; some regions predominantly expressed either the alpha1 or the alpha2 subunit (22) explaining mismatches between the alpha1 and beta1 distribution observed earlier (23). Interestingly, cellular colocalization of GC subunits with neuronal NO synthase was shown to occur in midbrain and cerebellum of the rat arguing for NO as an intracellular autocrine agent (24). Recently, the tissue distribution of GC subunits was quantitatively assessed in mice by real time PCR and Western blot. The alpha1/beta1 isoform was the dominant isoform in all tissues with the highest levels found in lung. The alpha2/beta1 isoform was found to be predominantly expressed in brain, which was the only tissue, in which the two isoforms were found in quantitatively similar amounts (25). The beta2 content of the tissues analyzed was negligible arguing against a relevant physiological role of this subunit in mice. Taken together, the two functionally indistinguishable isoforms of NO-sensitive GC are distributed differentially. The major occurrence in brain speaks in favor of the alpha2/beta1 as the neuronal isoform of NO-sensitive GC which participates in synaptic transmission. This notion is corroborated by the finding that this isoform is targeted to synaptic membranes. In contrast, the alpha1/beta1 isoform is most prominent in vascularized tissues and may therefore represent the vascular form of the enzyme. Thus, the differential subcellular and tissue localization of the two GC isoforms may reflect the association to the neuronal and endothelial NO synthases, respectively.

3.4. Regulation of NO-sensitive Guanylyl Cyclase

The identification of NO as the physiological activator of NO-sensitive GC was one of the key findings for its role as a signaling molecule in almost any tissue. Binding of NO to the prosthetic heme group of NO-sensitive GC can be seen as an on-switch for cGMP synthesis. This high affinity-binding leads to the formation of NO-heme complex and a subsequent conformational change is responsible for the up to 200-fold increase in the catalytic rate (26-28).

The precise steps involved in the formation of the five-coordinated nitrosyl-heme complex that finally induces the activation of the enzyme are currently being discussed (Figure 1). NO initially binds to the sixth coordinating position of the heme iron which, after the formation of a six-coordinate intermediate leads to breakage of the histidine-iron bond and, thus, a five-coordinated nitrosyl-heme complex. Scission of the histidine-iron bond appears to be the crucial step for activation since a six-coordinated nitrosyl adduct does not activate the enzyme (29, 30). The six-coordinate complex is an intermediate complex with NO and the His residue binding the heme iron; conversion to the five-coordinated (nitrosyl-heme) complex, a step at least three orders of magnitude slower than the initial NO binding step, is thought to occur in an NO-dependent manner.

The heme-containing cytochrome c' from denitrifying bacteria may serve as a model for NO-sensitive GC. Here, NO first binds to the distal sixth coordinating position of the heme. Then, a second NO molecule can bind to the proximal side thereby displacing the histidine and finally repelling the NO at the distal face (31). Although this is an appealing model, one has to bear in mind that the heme environments differ as e.g. the heme in cytochrome c', in contrast to GC, is covalently bound by cysteine-thioether linkages; nevertheless, both heme proteins share some properties as they both form five-coordinated NO- and six-coordinated CO-adducts and do not bind oxygen.

For cGMP synthesis to be switched-off,

deactivation of GC has to occur. Generally, dissociation of NO from the heme group is considered to lead to deactivation. Other five-coordinated heme proteins exhibit very slow NO dissociation rates (32); if this were the case for NO-sensitive GC, the enzyme would not be able to immediately respond to changing NO concentrations. Both, dissociation of NO as well as deactivation of the enzyme after the addition of NO scavengers have been investigated.

A half life of approx. 5 s for the NO-GC complex in the presence of GTP has been determined spectrophotometrically using oxyhemoglobin as NO scavenger (33). The substrate GTP seems to be crucial as in its absence the dissociation was greatly slowed down to a half life of 2 min (32, 34). As GTP is always present within a cell, the effect of substrate on NO dissociation is probably not regulatory; nevertheless GTP should be included in '*in vitro*' experiments to ensure the enzyme's natural behavior.

Deactivation of NO-sensitive GC was also measured by monitoring the catalytic rate of purified NO-stimulated GC after the addition of the NO scavenger oxyhemoglobin (35). The determined half life of approximately 4 s is in good accordance with the spectrophotometrically measured NO dissociation rate in the presence of GTP. In sum, at least for the purified enzyme, NO dissociation and deactivation appear to be parallel processes. The deactivation assessed in cytosolic preparations of bovine retina yielded a half life of 18 s at 20°C, which can be extrapolated to a half life of 5 s at 37°C (36). In intact cerebellar cells, a 25-fold faster deactivation with a half life of 0.2 s has been reported (37). Similar results have been obtained in human platelets (35). The reason for the much faster deactivation in intact cells compared to purified or cytosolic preparations remains unclear.

3.5. NO sensitizers

A group of substances has been identified during the last years which sensitize GC towards its physiological activator NO. The first compound to be identified, the benzylindazole YC-1, has been originally published as inhibitor of platelet aggregation (38). An approx. 10-fold activation of GC by YC-1 has been demonstrated and, since it was not be prevented by NO scavengers, this activation has been described as NO-independent (39). Yet more impressive, YC-1 shifted the NO concentration response curve by one order of magnitude to the left indicating sensitization of NO-sensitive GC towards its activator. Moreover, YC-1 turned CO into an effective GC activator as in the presence of YC-1, CO stimulated the enzyme almost to the same extent as NO. YC-1 analogues also acting as NO sensitisers have been developed which generally feature YC-1-like properties and differ mainly in the EC_{50} (40, 41).

Inhibition of deactivation has been suggested as the underlying mechanism of the YC-1 sensitizing effect (39) and the issue has been studied in detail with purified GC (35). In the presence of YC-1, the fast deactivation (half life 4 s) was prolonged 150-fold yielding a half life in the range of 10 min. Moreover, deactivation of a GC mutant which forms cAMP instead of cGMP (substitution of three amino acids in the catalytic domain) was not inhibited by YC-1; this mutant also lacked the YC-1-induced sensitization suggesting that the sensitization results from the inhibition of deactivation.

YC-1 is able to bind to the heme-depleted enzyme indicating the existence of an allosteric site, yet, YC-1 stimulation requires the presence of the heme group (42). The exact binding site of YC-1 and other new analogues is still unclear. Several studies attempted to identify the binding site of YC-1 by either photoaffinity labeling (40), resonance Raman studies (43) or mutational analysis (44, 35). In sum, clear evidence about the YC-1 binding site is still missing.

It should be emphasized that the key compound of this group, YC-1, not only acts as a direct activator of NO-sensitive GC but also exhibits a variety of other mechanism of action. Of importance within NO/cGMP signaling, YC-1 inhibits the cGMP-degrading phosphodiesterases such as PDE5 and PDE1 (45, 46). In platelets, the inhibition of PDE5 by YC-1 led to a drastic, over 1,000-fold increase in cGMP in the presence of NO (45). Due to the high catalytic rate of PDE5, some of the YC-1 effects observed in intact cells may well be caused by the inhibition of cGMP degradation rather than solely by the stimulation of NO-sensitive GC. Additional cGMP-independent effects of YC-1 have been reported as induction of hypoxia-inducible factor-1a, reduction of Ca^{2+} fluxes, inhibition of superoxide production or prevention of NO-induced neurotoxicity, the latter probably via sodium channel inhibition (for review, see 47).

In addition to the NO sensitizers, new heme independent activators of GC have been identified. These compounds stimulate the heme-free rather than the heme-containing enzyme suggesting substitution of the heme group as the underlying mechanism of action (48).

3.6. Inhibitors

Two substances have been shown to specifically inhibit the NO-sensitive GC. These are the quinoxalin derivative 1H-[1,2,4]oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ) and the related NS 2028 (oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one) (49, 50). The inhibitory effect has been demonstrated in a variety of tissues (49, 51-53). ODQ binds in an NO-competitive manner and inhibits NO-stimulated activity leaving basal enzymatic activity unchanged. Oxidation of the heme iron has been identified as the underlying mechanism (54, 55). Methylene blue and LY-83583 act as unspecific inhibitors of NO-sensitive GC; yet, they have been shown to also interfere with NO synthesis (56) or affect nucleotide-gated ion channels (57).

4. NEGATIVE FEEDBACK INHIBITION IN NO/cGMP SIGNALING

4.1. cGMP dynamics in intact cells: Role of PDE5

As described in the previous sections the alpha1/beta1 heterodimer of NO-sensitive GC is abundantly expressed in platelets and smooth muscle and likely represents the predominant isoform in the cardiovascular system.

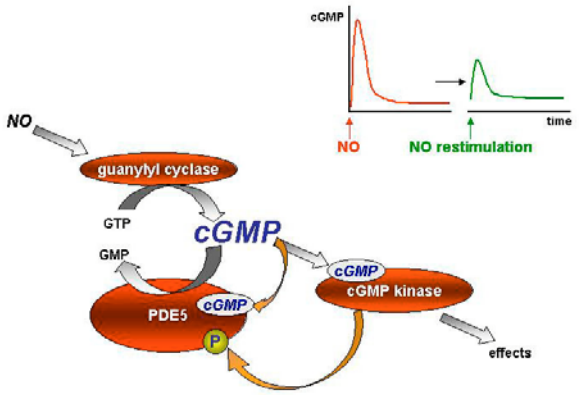

Figure 2. 'Feed-back' regulation within NO/cGMP signaling. Stimulation of NO-sensitive guanylyl cyclase results in an increase in the intracellular cGMP concentration. Parallel to the activation of cGMP kinase, cGMP directly activates PDE5 thus increasing its own degradation. In addition, PDE5 is phosphorylated by cGMP kinase which, in turn, increases the affinity of the GAF domains for cGMP. The cGMP-induced activation of PDE5 plays a major role in the desensitization of this signaling cascade: As an example, stimulation of platelets with NO leads to a reduced cGMP response upon a second (re)stimulation. For further information see text

Here, this isoform is responsible for the anti-aggregatory and relaxing effects of NO, respectively. In an intact cell, the intracellular cGMP concentration is determined by the ratio of synthesis by GCs and breakdown by cGMP-degrading PDEs. In platelets and smooth muscle, a major determinant of cGMP degradation is the cGMP-specific cGMP-binding PDE (PDE5). The dominant role of PDE activity for the control of intracellular cGMP levels in platelets becomes evident when NO-sensitive GC is stimulated and PDE5 is inhibited by sildenafil. Under these conditions, cGMP accumulates to intracellular concentrations of 600-800 µM (e.g. in platelets) which appear to be exclusively limited by the availability of the substrate GTP (58). In the absence of any PDE inhibitor, the NO-induced cGMP response in platelets and aortic smooth muscle displays a spike-like profile (Figure 2). This only transient elevation of intracellular cGMP reflects dynamic changes in the ratio of cGMP formation and degradation. The contributions of enzymatic activities that are responsible for the shaping of the transient cGMP response have been analyzed in detail in platelets. In human platelets, a maximally active concentration of an NO donor causes a fast rise in the intracellular cGMP concentration to values of 50-100 µM within a few seconds; subsequently, cGMP returns to almost resting levels after 30-40 s. Remarkably, in murine and rat platelets the NO-induced rise and decline of intraplatelet cGMP are accomplished within 10-15 s and peak concentrations of 100-200 µM of intracellular cGMP are reached indicating even faster changes in cGMP formation and degradation in these species (59, 60).

The activities of NO-sensitive GC and PDE5 that govern the spike-like cGMP response have been analyzed using different approaches. In order to unmask the prevailing activity of NO-sensitive GC during the NO-induced cGMP response, PDE5 was inhibited by sildenafil at the different phases of the spike-like response. In a first report using human platelets, the velocity of cGMP synthesis remained unaltered during all phases of the cGMP response (58); conversely, in another report using rat platelets, the rate of cGMP formation declined with time to approximately 20 % of the maximal rate indicating desensitization of NO-sensitive GC (60). The major difference between the measurements lay in the value for the maximal rate, i.e. the reference. In rat platelets, the maximal rate was considerably faster than in human platelets; to date, there is no obvious explanation for this discrepancy of results other than variations between species.

In a second approach, human platelets were lysed at the different phases of the cGMP response and activity of NO-sensitive GC was determined *in vitro*. Here, no change in maximal activity of NO-sensitive GC was detected and the measured rates were in reasonable agreement with those previously determined in the intact human platelets. In fact, a compelling evidence for the existence of a desensitized NO-sensitive GC in lysates of cells is still lacking, which may be due to the existence of an essential cellular factor that is lost or diluted upon destruction of cell integrity.

On the other hand, an activation of PDE5 was found after NO-stimulation of intact human platelets. As this effect could be detected in the lysate after the breaking up of platelets it was assumed to be the result of a stable modification of the enzyme. In sum, the spike-like shape of the NO-induced cGMP response appears to be crucially governed by the downstream activation of PDE5 which thus represents a negative feedback regulation in NO/cGMP signal transduction.

4.2. Mechanism of NO/cGMP-induced activation of PDE5

Since PDE5 is an established target for PKG, phosphorylation was postulated as mechanism of PDE5 activation by several groups (61, 62, 58). So far, phosphorylation of PDE5 was demonstrated to occur in response to cGMP elevations in smooth muscle, platelets, cerebellar Purkinje neurons and T84 colonic carcinoma cells (58, 61-65). Although PDE5 can be phosphorylated by the catalytic subunits of PKA *in vitro* it is usually catalyzed by PKG in intact cells as demonstrated using tissue from PKG knock-out mice (59, 62, 64). In intact cells, phosphorylation of PDE5 was shown to parallel NO/cGMP-induced activation in platelets and vascular smooth muscle and a reasonable correlation was observed in the time-courses of deactivation and dephosphorylation. Although an intriguing idea, phosphorylation of PDE5 has recently been shown not to represent the mechanism of activation; rather, an allosteric activation was shown to be induced directly by cGMP.

It has been known since the early 1990's that the cGMP-binding cGMP-specific PDE5 was able to bind at least one molecule cGMP per monomer to its non-catalytic

GAF-domains (66). Homologous GAF-domains (GAF-A and GAF-B) can also be found PDE2 where the binding of cGMP induces allosteric activation of the enzyme (67, 68). However, no functional consequences were observed upon cGMP binding to PDE5. Yet, the binding of cGMP was shown to be a prerequisite for the phosphorylation of PDE5 *in vitro* (69, 70), most likely due to the induction of a conformational change leading to the exposure of the phosphorylation site. An unequivocal activation and phosphorylation of PDE5 could only be demonstrated in intact cells after agonist-induced cGMP elevation or the use of cell-permeable cGMP analogues. In these experiments, a discrimination between activation and phosphorylation of PDE5 was virtually impossible to achieve (58, 62, 63, 65). Recently, the binding of cGMP to the regulatory GAF-A domain has been shown to induce an activation of PDE5 that is independent of phosphorylation (59, 71, 72). This model was further supported by the fact that binding of the competitive inhibitor sildenafil was enhanced in the presence of cGMP confirming a reciprocal communication between the catalytic domain and the regulatory GAF domain (73). After longer storage periods enzyme preparations appear to lose their ability to be activated by cGMP which may be attributed to the loss of an auto-inhibited conformation that relaxes into an activated state by itself, even in the absence of cGMP (72). The resulting loss in responsiveness to cGMP most likely represents the reason why a functional effect of cGMP binding on catalysis had never been observed in the past; usually, enzyme preparations were used after lengthy purification procedures and storage periods.

PDE5 phosphorylation appears to affect the stability of the activated cGMP-bound conformation of the enzyme. The cGMP binding affinity of the isolated regulatory domain of PDE5 was shown to be enhanced by phosphorylation reducing the k_d-value by 10-fold (74). These observations are consistent with the very slow deactivation of PDE5 observed in intact platelets after a short incubation with NO. Also *in vitro*, phosphorylation greatly reduced the rate of deactivation of PDE5 holoenzyme in lysates of transfected HEK cells (75). The physiological significance and the dynamic regulation of PDE5 phosphorylation remain to be established *in vivo*, but it is conceivable that phosphorylation acts as a memory for elevated cGMP limiting the sensitivity of the signaling pathway for incoming NO-signals (Figure 2).

4.3. Temporal regulation of PDE5 activity

As discussed above, the NO/cGMP-mediated allosteric activation of PDE5 critically determines the shape of the cGMP response in platelets and acts as a negative feedback regulation in the signaling pathway. Consequently, the efficiency of further incoming NO signals, i.e. the sensitivity of the signaling pathway, will also crucially depend on the activation status of PDE5. Therefore, the duration of PDE5 activation is another important parameter in the negative feedback control of NO/cGMP signaling in intact cells. In platelets, PDE5 activation induced by preincubation with a low physiological concentration of NO was shown to persist for at least 1 hour. Subsequent NO signals only elicited a reduced cGMP-response demonstrating a sustained desensitization of the signaling pathway. A rapid and sustained increase in PDE5 activity that causes desensitization thus represents an adaptation of the signaling pathway to elevated NO concentrations. NO/cGMP-induced desensitization was also found in rat aortic strips and HEK293 cells stably transfected with NO-sensitive GC and PDE5 (75). In vascular smooth muscle, the activation of PDE5 may also affect natriuretic peptide-mediated signaling via receptor-coupled guanylyl cyclases, which will be discussed below. In parallel to the NO/cGMP-mediated activation of PDE5, a PKG-mediated phosphorylation was also detectable in platelets for at least 1 hour after removal of the NO signal. As phosphorylation appears to stabilize the activated conformation of PDE5 it conceivably extends the duration of activation. Since cGMP binding is a prerequisite for phosphorylation, the phosphorylation status of PDE5 appears to be a good indicator for activation and thus for the sensitivity of GC/cGMP-signaling; however, the overall physiological consequences of PDE5 phosphorylation remain speculative.

4.4. Sensitization of NO/cGMP signaling

In a addition to the NO-induced negative feedback in the signaling pathway that leads to a sustained desensitization the opposite phenomenon, that is sensitization, has been observed in different experimental approaches. A so-called supersensitivity can be induced by decreasing the tonic NO production in the endothelium of blood vessels which is the main source of NO in the cardiovascular system. NO-shortage can be induced pharmacologically by inhibition of NO synthase or by targeted disruption of the gene coding for eNOS; the results obtained using either approach were consistent. Originally, the relaxation as well as the cGMP accumulation of arteries in response to exogenous NO were shown to be enhanced after treatment of aortic rings with NO-synthase inhibitors like L-NAME *ex vivo* (76). More recently, the results were confirmed using aortic and mesenteric segments from eNOS knock-out mice (77, 78) where the vasodilatory potency of NO-releasing agents was clearly enhanced. Consistent results were obtained after *in vivo* administration of the NOS inhibitor L-NAME in rats. In the treated animals, the acute effect of intravenous delivery of SNP on mean arterial pressure was tripled and cGMP accumulation was potentiated. Importantly, expression of α and β subunit of NO-sensitive GC was unaltered in aortae from eNOS knock-out mice and L-NAME-treated rats which argues against a transcriptional or translational regulation of NO-sensitive GC in response to decreased NO levels *in vivo* (77, 79).

Interestingly, in the study of Madhani and co-workers (78) the potency of natriuretic peptides that act by stimulation of particulate guanylyl cyclases (pGC) was also enhanced in vessels lacking eNOS. When the eNOS was pharmacologically inhibited for 30 min prior to the experiments in vessels from WT animals, hypersensitivity to both NO and natriuretic peptides was observed. Moreover, pretreatment of vessels with NO or natriuretic peptide induced the opposite effect, that is desensitization as well as cross-desensitization. These data demonstrate

coupling of NO/NO-sensitive GC signaling and NP/pGC signaling in mammalian arteries, which can only be explained by a common regulation. As both pathways converge on the level of cGMP degradation, the concept of sensitization/desensitization by modulation of PDE5 activity is an intriguing possibility to unify the data reported independently by different groups. Of course, the possibility of a regulation on the level of downstream effector systems cannot be ruled out. However, the vasodilator potency of the PDE-resistant direct PKG activator 8-pCPT-cGMP was unaltered in arteries from mice lacking eNOS (77) arguing against a mechanism downstream of cGMP. In addition, activation and phosphorylation of PDE5 was also observed in smooth muscle cells and T84 colonic carcinoma cells following activation of pGCs by natriuretic peptides or heat-stable enterotoxins, respectively (63, 65). The activation of PDE5 was also associated with a desensitization of the cGMP signaling pathway in T84 cells. Taken together, it appears reasonable to assume that the tuning of PDE5 activity represents a regulatory link between signaling through NO-sensitive GC and pGC in cells expressing both types of cyclases and therefore crucially determines the overall sensitivity of hormone-induced cGMP signaling.

5. ACKNOWLEDGMENT

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