Direct activation of PDE5 by cGMP: long-term effects within NO/cGMP signaling

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In platelets, the nitric oxide (NO)–induced cGMP response is indicative of a highly regulated interplay of cGMP formation and cGMP degradation. Recently, we showed that within the NO-induced cGMP response in human platelets, activation and phosphorylation of phosphodiesterase type 5 (PDE5) occurred. Here, we identify cyclic GMP-dependent protein kinase I as the kinase responsible for the NO-induced PDE5 phosphorylation. However, we demonstrate that cGMP can directly activate PDE5 without phosphorylation in platelet cytosol, most likely via binding to the regulatory GAF domains. The reversal of activation was slow, and was not completed after 60 min. Phosphorylation enhanced the cGMP-induced activation, allowing it to occur at lower cGMP concentrations. Also, in intact platelets, a sustained NO-induced activation of PDE5 for as long as 60 min was detected. Finally, the long-term desensitization of the cGMP response induced by a low NO concentration reveals the physiological relevance of the PDE5 activation within NO/cGMP signaling. In sum, we suggest NO-induced activation and phosphorylation of PDE5 as the mechanism for a long-lasting negative feedback loop shaping the cGMP response in human platelets in order to adapt to the amount of NO available.

Introduction

The signaling molecule nitric oxide (NO)* plays an important role in the cardiovascular and nervous system under physiological and pathophysiological conditions. An important function of NO is the activation of NO-sensitive guanylyl cyclase through its binding to the prosthetic heme group of the enzyme. The resulting activation of guanylyl cyclase leads to an enhanced conversion of GTP to cGMP (Koesling and Friebe, 1999). An elevated intracellular cGMP concentration mediates a variety of cellular and physiological responses, depending on the cell type and tissue. In the vascular system, the relaxation of blood vessels and the inhibition of platelet aggregation are mediated by cGMP (Schwarz et al., 2001). Cyclic GMP exerts its effects through activation of different effectors, such as cGMP-gated ion channels (Biel et al., 1999), cGMP-dependent protein kinases (Lohmann et al., 1997; Hofmann et al., 2000) and cGMP-regulated phosphodiesterases (PDEs; Juilfs et al., 1999; Francis et al., 2000).

In addition to cGMP formation, degradation of cGMP by cyclic nucleotide PDEs determines intracellular cGMP levels. Several different isoforms of PDEs are known to hydrolyze cGMP, and are therefore potentially involved in controlling the intracellular cGMP turnover. In platelets and smooth muscle cells, an important regulator of cGMP levels is the cGMP-binding cGMP-specific PDE (PDE5). The enzyme is a homodimer with two regulatory, putative cGMP-binding GAF domains (found in cGMP-phosphodiesterases, adenylyl cyclases, and Escherichia coli FhlA) and one catalytic site per monomer (Corbin and Francis, 1999); each monomer contains a phosphorylation site that is conserved across species, e.g., in human, rat, bovine, canine, and murine isoforms. PDE5 has been shown to be phosphorylated in vitro by cyclic GMP-dependent protein kinase I (cGKI) and cyclic AMP-dependent protein kinase (cAK; Corbin et al., 2000). Phosphorylation of PDE5 requires binding of cGMP to the

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*Abbreviations used in this paper: cGKI, cyclic GMP-dependent protein kinase I; cAK, cyclic AMP-dependent protein kinase; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PDE, phosphodiesterase; VASP, vasodilator-activated stimulated phosphoprotein; WT, wild-type.

Key words: cyclic GMP; cGMP-dependent protein kinase; GAF domain; guanylyl cyclase; platelets
regulatory domains (Turko et al., 1998), and has been suggested to enhance cGMP hydrolysis in vitro and in intact cells (Wyatt et al., 1998; Rybalkin et al., 2001). An increase in PDE5 activity induced by cGKI-mediated phosphorylation potentially represents an important feedback mechanism to limit amplitude and duration of a cGMP signal in cells that express this PDE isoform.

However, the functional relevance of PDE5 activation and phosphorylation within the NO/cGMP signaling pathway has not yet been thoroughly investigated. In platelets, we have shown that NO leads to a rapid, biphasic cGMP response that is indicative of a tight regulation of cGMP-forming and -degrading activities (Mullershausen et al., 2001). Furthermore, preincubation of platelets with NO rapidly led to a reduction of the NO-induced cGMP response, revealing short-term desensitization occurring within the NO/cGMP signaling pathway. Although cGMP formation by guanylyl cyclase remained unaltered during the entire course of the cGMP response, the activity of PDE5 was found to be enhanced in NO-incubated platelets (Mullershausen et al., 2001). Therefore, the rapid NO-induced desensitization of the system has been attributed to an enhanced cGMP degradation through activation of PDE5. With antibodies specific for the phosphorylated form of PDE5, the activation was shown to be paralleled by phosphorylation. The components of the signaling pathway that act downstream of NO and cause the activation and phosphorylation of PDE5 in human platelets are so far unknown. Moreover, the reversal of PDE5 activation and phosphorylation has not been investigated in intact cells.

In the present paper, we identify the components of the NO/cGMP signaling pathway that mediate PDE5 activation and phosphorylation in response to NO in intact platelets. We demonstrate that cGMP by itself is able to activate PDE5, most likely by binding to the GAF domains of the enzyme, and we supply evidence that phosphorylation enhances the activation induced by cGMP. By monitoring the decline in activity in platelet supernatant and in intact platelets, we show that the NO-induced PDE5 activation persists for over 1 h. In addition, we demonstrate that the relatively small increase in PDE activity induced by a physiologically occurring NO concentration is sufficient to reduce the NO-induced cGMP response for as long as 1 h.

Results

NO-induced activation and phosphorylation of PDE5 in platelets depends on guanylyl cyclase stimulation

In platelets, NO is known to cause inhibition of aggregation by increasing the intracellular cGMP content and the subsequent activation of cGMP-dependent protein kinase. In these cells, PDE5 has been shown to be the relevant PDE for cGMP degradation; recently, we showed that the cGMP response induced by the NO-releasing agent GSNO was paralleled by the activation and phosphorylation of PDE5 in the supernatant prepared from intact NO-incubated platelets (Mullershausen et al., 2001). Here, we used the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), to find out whether the NO effects on PDE5 depended on guanylyl cyclase-catalyzed cGMP formation, and measured cytosolic PDE activity after incubation of intact platelets with 1 μM of the NO donor DEA-NO in the absence and presence of the guanylyl cyclase inhibitor ODQ (20 μM), then platelets were lysed and PDE activity was determined in the cytosolic fractions. Data represent means ± SD of three independent determinations in duplicate. (A) The DEA-NO–induced phosphorylation of PDE5 was detected in a Western blot; PDE5 content of the samples is also shown (top). The band seen below phospho-PDE5 is due to nonspecific binding of the antibody in human platelet samples. Bottom shows Western blot detection of phosphorylated VASP.

Figure 1. NO-induced activation and phosphorylation of PDE5 is inhibited by ODQ. (A) Human platelets were incubated for 2 min without or with 1 μM DEA-NO in the absence and presence of the guanylyl cyclase inhibitor ODQ (20 μM), then platelets were lysed and PDE activity was determined in the cytosolic fractions. Data represent means ± SD of three independent determinations in duplicate. (B) The DEA-NO–induced phosphorylation of PDE5 was detected in a Western blot; PDE5 content of the samples is also shown (top). The band seen below phospho-PDE5 is due to nonspecific binding of the antibody in human platelet samples. Bottom shows Western blot detection of phosphorylated VASP.
Removal of cGKI reduces cGMP-induced phosphorylation and activation of PDE5 in platelet supernatant

As guanylyl cyclase–catalyzed cGMP formation was required for the NO-induced activation and phosphorylation of PDE5, we tested whether these effects could be induced by cGMP in broken platelet preparations. In these experiments, platelet supernatants were preincubated with 100 μM cGMP. Subsequently, in this and all following cGMP preincubation experiments, platelet supernatants were diluted to a final cGMP concentration below at least 0.1 μM, and PDE5 activity was then determined with 1 μM cGMP as substrate. As shown in Fig. 2 B (bar 1) and Fig. 2 C (lane 1), incubation of lysed platelets with cGMP led to activation (1.9-fold) and phosphorylation of PDE5.

Next, we investigated the role of cGKI in the process of phosphorylation and activation of PDE5 in platelet supernatants. To quantitatively remove cGKI, we precipitated the enzyme from the platelet supernatant using Rp-8-AET-cGMPS coupled to agarose beads. With immunoblot analysis (Fig. 2 A, top), we confirmed that the majority of cGKI had been cleared from the sample (lane 1 vs. lane 2); lane 3 shows the signal after reconstitution with 0.2 μg exogenous cGKI. Treatment with Rp-8-AET-cGMPS-agarose did not lead to a loss of PDE5 activity in the supernatant (unpublished data), demonstrating that PDE5 was not precipitated. Moreover, equal amounts of PDE5 were present in the samples as controlled by immunoblot analysis (Fig. 2 A, bottom).

The cGKI-containing, -depleted, and -reconstituted supernatants were preincubated with 100 μM cGMP for 5 min. The resulting PDE activities are shown in Fig. 2 B. Compared with the 1.9-fold activation of PDE5 in the cGKI-containing supernatant, the cGMP-induced activation was only 1.2-fold in the cGKI-depleted sample. On reconstitution with purified cGKI, the cGMP-induced activation was restored (2.2-fold). The respective phosphorylation

Figure 2. cGKI-mediated phosphorylation and activation of PDE5. (A) Platelet supernatant was incubated with 8-AET-cGMPS-agarose to remove cGKI. Immunoblot analysis using an anti-cGKI antibody (top) is shown after incubation of the supernatant with control agarose (lane 1, cGK-containing), the cGMP-agarose (lane 2, cGK-depleted) and after reconstitution of the cGMP-agarose–treated supernatant with recombinant cGKI (lane 3, cGK-reconstituted). An anti-PDE5 antiserum (bottom) was used to control PDE5 content after incubation with cGMP-agarose (B) The obtained supernatants were preincubated with 100 μM cGMP for 5 min, diluted, and PDE activity was subsequently determined. Data represent means ± SD of three independent experiments performed in duplicate. (C) Phosphorylation of PDE5 and VASP was detected after the preincubation with 100 μM cGMP using the respective phospho-specific antibodies in Western blot analysis.

Figure 3. NO-induced cGMP signaling in platelets from cGKI−/− mice. (A) Platelets from cGKI−/− or WT mice were incubated with 300 μM GSNO, and the cGMP content was determined at the indicated time points. Shown are means of three independent experiments with platelets from three WT and three cGKI−/− mice. (B) Western blot analysis of untreated and NO-treated platelets (300 μM GSNO; 1 min) using phospho-PDE5 antibody (top) and total VASP serum (bottom) are shown. In contrast to the phospho-VASP antibody, the total VASP serum recognizes unphosphorylated and phosphorylated VASP. A shift toward higher molecular weight indicates phosphorylation at Ser-157.
of PDE5 and VASP is shown in Fig. 2 C. These data suggest that cGKI is responsible for phosphorylation, apparently promoting the activation of PDE5 in platelets.

**NO-induced cGMP response in platelets from cGKI/−/− mice**

To further investigate the role of the cGKI-mediated phosphorylation of PDE5 within the NO-induced cGMP response, we used platelets from cGKI-deficient mice. Fig. 3 A shows the biphasic cGMP response of intact platelets from wild-type (WT) and cGKI/−/− mice. Stimulation with 300 μM GSNO led to a cGMP response similar to that seen in human platelets, although remarkably, the duration of the cGMP transient in mice was even shorter. However, the human platelets, although remarkably, the duration of the cGMP response (for control, see Fig. 5 A). First, cGKI was precipitated from platelet supernatant as described above. The supernatant was diluted 20-fold, and subsequently was treated with alkaline phosphatase to degrade ATP. Then, the diluted, cGKI-depleted platelet supernatant was incubated without or with 50 μM cGMP for 1 min at 37°C in the absence of ATP and in the presence of 100 μM Rp-8-Br-cAMPS to inhibit cAK. cGMP preincubation was terminated by a further 250-fold dilution into ice-cold dilution buffer (bar graph, showing maximal cGMP-induced activation) or prewarmed buffer (37°C). Aliquots were withdrawn and assayed for PDE5 activity at the indicated time points (line graph).

**cGMP-induced activation of PDE5 occurs independent of phosphorylation**

Thus, we further investigated whether phosphorylation is required for the observed activation of PDE5 using a platelet supernatant in which phosphorylation of PDE5 did not occur (for control, see Fig. 5 A). First, cGKI was precipitated from platelet supernatant as described above. The supernatant was diluted 20-fold, and subsequently was treated with alkaline phosphatase to degrade ATP. Then, the diluted, cGKI- and ATP-depleted supernatant was incubated with cGMP (50 μM for 1 min), and diluted again before measurement of PDE activity (1 μM substrate). Surprisingly, PDE5 activity was enhanced 2.3-fold in the cGMP-preincubated sample as shown in Fig. 4, suggesting that cGMP by itself is able to activate PDE5.

To monitor the time course of the reversal of cGMP-induced activation, the cGMP-preincubated supernatant was diluted 250-fold (to <0.2 μM cGMP) and was further incubated at 37°C. At the indicated time points, aliquots were withdrawn and PDE activity was measured. As shown in Fig. 4, the decline in PDE activity was unexpectedly slow. Increased PDE activity compared with control was still observed after 60 min. These data suggest that cGMP binding to the regulatory domain has a long-term stimulatory effect on the catalytic rate of PDE5.

**Phosphorylation enhances cGMP-induced activation of PDE5**

However, a 100-μM cGMP preincubation of the undiluted supernatant only caused a significant activation of PDE5 in the presence of cGKI (1.8-fold vs. 1.2-fold; shown in Fig. 5 A), clearly indicating a role of the phosphorylation in the observed activation. Although in this experiment, preincubation (5 min) was started with 100 μM cGMP, cGMP was completely degraded within 2 min of preincubation due to the high PDE activity in the undiluted supernatant (unpublished data). So, we hypothesized that phosphorylation enhanced cGMP-induced PDE5 activation, permitting activation to occur at lower cGMP concentrations. In this case, a higher cGMP concentration during preincubation should result in PDE5 activation even in the absence of cGKI. Indeed, phosphorylation of the cGKI-depleted supernatant with 500 μM cGMP resulted in a 2.5-fold PDE5 activation that was almost as pronounced as that seen in the cGKI-containing supernatant (2.9-fold; Fig. 5 A). In this experiment, 150 μM residual cGMP was measured after 5 min of preincubation (unpublished data). These results suggest that the effect of phosphorylation can at least be partially mimicked by high cGMP concentrations.

In these experiments, the absence of PDE5 phosphorylation was controlled in the Western blot in Fig. 5 A. Clearly, no phosphorylation of PDE5 occurred in the cGKI- and ATP-depleted samples; this experiment also represents the appropriate control for the results shown in Fig. 4.

In addition to the activation, we monitored the time course of deactivation of the phosphorylated and unphosphorylated PDE5. Again, activation was sustained and still detectable after 60 min (Fig. 5 B). In this experimental setting, no significant difference in the time courses of deactivation between the phosphorylated and unphosphorylated PDE5 could be observed. In sum, the data suggest that the cGMP-induced activation of PDE5 does not require (but is enhanced by) phosphorylation.
The NO-induced PDE5 activation and phosphorylation persists after scavenging NO

The experiments in platelet supernatant showed that PDE5 activation is very sustained and reverses only slowly in vitro. Next, we studied the time course of the NO-stimulated activity of PDE5 in intact cells. Intact platelets were further incubated at 37°C. At the indicated time points, aliquots were withdrawn, platelets were lysed, and cytosolic PDE activities were measured (Fig. 6 A, primary axis). Scavenging of NO resulted in marked reduction of PDE5 activity within 10 min (to ~1.8-fold over basal). However, after 10 min, the decline in PDE activity decelerated and a 1.5-fold activation of PDE5 was still detectable 60 min after the removal of NO. The concomitant phosphorylation of PDE5 is presented as Western blot in Fig. 6 B; the quantitative analysis of the immunoreactive signals displayed in Fig. 6 A (secondary axis) shows that the apparent kinetics of dephosphorylation are in good accordance with PDE5 deactivation. A substantial degree of PDE5 phosphorylation was still detected 60 min after addition of the NO scavenger. These data demonstrate that the NO-induced activation and phosphorylation of PDE5 is sustained in intact cells. Thus, a short NO signal is likely to lead to a long-lasting desensitization of the NO/cGMP signaling pathway.

Figure 5. Comparison of activation and its reversal of phosphorylated vs. nonphosphorylated PDE5. cGK-containing and -depleted platelet supernatants as described for Fig. 2 were preincubated with 100 or 500 μM cGMP for 5 min at 37°C in the presence or absence of ATP, respectively. 100 μM Rp-8-Br-cAMPS was present in both samples to inhibit cAK. (A) cGMP-induced activation of PDE5. The Western blot shows the concomitant phosphorylation of PDE5. (B) After the preincubation, samples were diluted 1,000-fold into dilution buffer and further incubated at 37°C. Aliquots were withdrawn and assayed for PDE5 activity at the indicated time points. Data represent means ± SD of seven independent determinations using four different cytosolic preparations.

Figure 6. Reversal of NO-induced PDE5 activation and phosphorylation in intact platelets. Intact human platelets were incubated with 1 μM DEA-NO for 5 min, and then a 50-fold excess of the NO scavenger carboxy-PTIO (50 μM) was added. Aliquots were withdrawn at the indicated time points and lysed. (A) PDE activity was determined (circles, primary axis) and PDE5 phosphorylation was assessed using quantitative Western blot analysis (squares, secondary axis). Data represent means of 10 samples assayed for PDE activity and quantitative analysis of six Western blots, respectively. (B) Representative Western blot for detection of PDE5 phosphorylation (top) after the addition of carboxy-PTIO; PDE5 content of the samples was controlled using a PDE5 antibody (bottom).
Long-term desensitization of the cGMP response in platelets

To verify the proposed long-term desensitization of the cGMP response induced by NO, we looked at the cGMP response in intact platelets. In our recent work, we have described a reduced cGMP response after 3 min of preincubation with NO (Mullershausen et al., 2001). Here, we monitored the desensitization of the cGMP response 1 h after a single, comparatively low, physiologically occurring NO stimulus (100 nM DEA-NO). As can be seen in Fig. 7 A (inset), the initial NO-induced cGMP increase elicited by 100 nM DEA-NO only leads to a very minor increase in cGMP; for comparison, a maximal response (10 μM DEA-NO) is shown in the same graph. 1 h after this NO stimulus, platelets were restimulated with a maximally effective DEA-NO concentration (10 μM). Clearly, the cGMP response of the NO-preincubated platelets was reduced with peak cGMP levels, ~60% of those in the control. PDE activity was still elevated significantly (1.25-fold, P < 0.002; Fig. 7 B, bar graph), and phosphorylation of PDE5 was still detectable (Fig. 7 B, Western blot), which very likely accounts for the observed desensitization of the NO-induced cGMP response.

Discussion

In a previous report, we showed that NO elicits a fast biphasic cGMP response in human platelets and rat aorta. In platelets, cGMP reached peak levels within 5–10 s after NO stimulation; then, cGMP levels rapidly declined to almost basal levels after <40 s. This characteristic response indicated a complex interplay of cGMP formation and degradation. In fact, the transient elevation in intraplatelet cGMP was accompanied by activation and phosphorylation of PDE5 within a few seconds after NO stimulation. Moreover, preincubation with NO for 3 min caused desensitization of the signaling pathway as shown by a reduced cGMP response on NO restimulation.

In this paper, we show that the NO-induced activation and phosphorylation of PDE5 in human platelets was abolished in the presence of the guanylyl cyclase inhibitor ODQ, ruling out any other NO targets involved in PDE5 activation.

To identify further components involved in the signaling cascade, we used supernatants from lysed platelets instead of intact platelets. In experiments with whole platelet lysates, the possibility that PDE5 is associated with the platelet membranes and redistributes on NO stimulation had been ruled out (Mullershausen et al., 2001). In the supernatant, we were able to show that cGMP is sufficient to elicit activation and phosphorylation of PDE5. Removal of cGKI from the platelet supernatant caused a marked reduction of cGMP-induced PDE5 activation and phosphorylation, whereas the addition of purified cGKI restored the ability of cGMP to exert its effects on PDE5. These data suggested that cGKI mediates the activation of PDE5 via phosphorylation. However, the cGMP response in platelets from cGKI−/− mice did not differ from that in WT mice, and phosphorylation of PDE5, although to a substantially lower extent, was still observed. Apparently, in the cGKI−/− platelets, another kinase, most likely cAK cross-activated by cGMP, can phosphorylate PDE5 in response to NO. Direct activation of cAK by cGMP has been reported to occur at ~10 μM cGMP (Ogreid et al., 1985). Assuming a murine platelet volume of 3.3 femtoliters (Poole, 1996), intraplatelet cGMP concentrations transiently reach 50–100 μM after maximal NO stimulation. With cGMP bound to the regulatory domains of PDE5, cAK appears as a plausible candidate for PDE5 phosphorylation in cGKI-deficient platelets. However, the discrepancy of an unaltered cGMP response and
the reduced phosphorylation in platelets from cGKI−/− mice prompted us to further study the relationship between phosphorylation and activation of PDE5.

For that reason, we measured the cGMP-induced increase in PDE5 activity in a platelet supernatant in which phosphorylation of PDE5 could not occur. In this platelet supernatant, cGKI had been removed by precipitation, ATP had been degraded, and cGMP did not induce phosphorylation of PDE5 as controlled with the antibody against the phosphorylated enzyme. Moreover, before the actual experiment (i.e., preincubation with cGMP), the platelet supernatant was diluted 20-fold to impede phosphorylation by any residual cGKI being present before precipitation. Preincubation of this platelet supernatant with cGMP resulted in an approximately twofold increase in PDE5 activity. It is most tempting to speculate that the increase in catalytic rate is caused by binding of cGMP to the GAF domains of PDE5. Because increased PDE activity was sustained and still detectable 1 h after dilution, cGMP bound to the regulatory domains can be assumed to dissociate very slowly. Allosteric regulation of enzymatic activity on cyclic nucleotide binding to the evolutionarily highly conserved GAF domains has been demonstrated for the cGMP-stimulated cAMP-degrading PDE type 2 (Martins et al., 1982) and for a self-activating adenylyl cyclase class III from the cyanobacterium Anabaena (Kanacher et al., 2002). Although cGMP binding to the noncatalytic GAF domains of PDE5 has been extensively studied and described in the literature, a stimulatory effect on catalysis has so far not been demonstrated. Only recently, activation of recombinantly expressed PDE5 by cGMP binding to the GAF-A domain was demonstrated by Beavo’s laboratory (Rybalkin et al., 2003). The fact that the cGMP-induced PDE5 activation could now be detected was probably due to the use of fresh cytosolic preparations in each experiment because the ability of cGMP to activate PDE5 was lost on storage.

However, under some experimental conditions, phosphorylation of PDE5 appeared to be required for cGMP-mediated activation, as demonstrated in Figs. 2 and 5. These data suggested that phosphorylation facilitated cGMP-induced PDE5 activation, allowing it to occur at a cGMP concentration not sufficient to directly induce the activation. And indeed, a higher cGMP concentration in the same experimental setting did cause a phosphorylation-independent PDE5 activation (see Fig. 5 A). We conclude that cGMP alone can activate PDE5 and that this activation is facilitated by phosphorylation; whether this is due to an increase in affinity of the allosteric sites for cGMP or whether the stimulatory effect of bound cGMP is enhanced by phosphorylation cannot be decided in our experimental setting. Nevertheless, under physiological conditions, the NO/cGMP-induced activation and phosphorylation of PDE5 will occur in parallel.

To verify the physiological significance of the sustained activation of PDE5 in a cellular environment, we induced activation of PDE5 in intact platelets with NO and monitored PDE activity after scavenging residual NO. Although a significant decline in PDE5 activity was measurable after 10 min, elevated PDE5 activities were detected for up to 1 h, suggesting the increase in PDE5 activity as a mechanism sufficient for long-term desensitization. Our results are in good accordance with the results published by Wyatt et al. (1998), who found that ANP-elicited activation of PDE5 in primary vascular smooth muscle cells was still detectable after 30–60 min.

Finally, we studied whether the observed long-lasting increases in PDE5 activity seen in platelet supernatant are sufficient to cause reduction of the NO-induced cGMP response in intact platelets. Indeed, even 1 h after a single application of a physiologically occurring NO concentration, a marked reduction of the cGMP response toward a maximally effective NO concentration was observed. Therefore, the enhanced degradation of cGMP appears to represent the molecular basis for the prolonged desensitization of the NO/cGMP system.

It is tempting to speculate that the mechanism of PDE5 activation in response to NO is not limited to platelets, but also represents a general model for negative feedback regulation of the cellular cGMP turnover. This idea is supported by a report of PDE5 activation and phosphorylation in isolated vascular smooth muscle cells (Rybalkin et al., 2001). Thus, the mechanism of an enhanced cGMP breakdown by a sustained activation of PDE5 may very well explain the different states of NO sensitivities found in various studies, and may well contribute to the development of tolerance to NO-releasing compounds observed in vascular smooth muscle, a common problem in the treatment of coronary heart disease.

Materials and methods

Reagents

Recombinant cGKI and COOH-terminal anti-cGKI-α and -β antibody were purchased from Calbiochem-Novabiochem. Affinity-purified antibody to phosphorylated PDE5 was prepared as described previously (Liu et al., 2002). Antiserum to total VASP, mAb to Ser239-phosphorylated VASP, DEA-NO (2,2-diethyl-1-nitroso-oxadiazoline), GSNOS (N-nitrosoglutethione), ODQ, and carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) were purchased from Qbiogene. pAb to PDE5 was raised by immunizing rabbits with an NHS-terminal fragment of 30 KD human PDE5A1 coupled to GST. Agarose beads coupled to Rp-8-AET-cGMPs (8-camomoylthioguanosine-3’,5’-cyclic monophosphate; Rp-isomer) and AET, and Rp-8-Br-cAMPS were obtained from Biolog. α[32P]GTP was purchased from Amersham Biosciences. Soluble guanylyl cyclase was purified from bovine lung as described previously (Humbert et al., 1990). All other reagents were purchased from Sigma-Aldrich.

Isolation of human platelets

Collection of blood and isolation of human platelets was performed largely as described previously (Freibie et al., 1998). In brief, 20 ml of blood was drawn into 4 ml ACD (85 mM trisodium-citrate, 65 mM citric acid, and 100 mM glucose) and centrifuged for 10 min at 300 g. Platelet-rich plasma was collected and recentrifuged for 5 min at 1,500 g and 20°C. The platelet pellet was carefully resuspended in platelet buffer (150 mM NaCl, 0.55 mM NaH2PO4, 7 mM NaHCO3, 2.7 mM KCl, 0.5 mM MgCl2, 5 mM Hepes, pH 7.4, and 5.6 mM glucose). Suspensions were allowed to rest at RT for 60 min before the start of the experiments.

Measurement of PDE activity in the supernatant fraction after NO incubation of intact platelets

Platelet suspensions were adjusted to 3 × 109 platelets/ml. Aliquots of 450 μl were equilibrated at 37°C for 10 min, and were stimulated with DEA-NO. Incubation time, NO donor, and other substances were applied as indicated. After the incubation, platelets were lysed by the addition of 500 μl of an ice-cold protease inhibitor cocktail (2 μM pepstatin A, 0.4 μM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride and 2 mM sodium...
vanadate, 1 mg/ml BSA, and 4 mM DTT) and brief sonication (1 pulse of 5 s) on ice using a sonifier (model B-12; Branson). After centrifugation (for 15 min at 20,000 g and 4°C), PDE activity in the supernatant was measured by the conversion of [32P]GMP (synthesized from α-[32P]GTP using purified guanylyl cyclase) to guanosine and [32P]phosphate in the presence of alcaline phosphatase at 37°C for 10 min. Reaction mixtures contained 1 μM of the supernatants, 100 μM MgCl2, 12 μM cGMP, 1 mM ATP, 0.1 mM CaCl2, 3 mM DTT, 0.5 mg/ml BSA, 2 U of alkaline phosphatase, and 50 mM triethyl- anolamine/HCl, pH 7.4, in a total volume of 0.1 ml. Reactions were stopped by the addition of 900 μl ice-cold charcoal suspension (20% activated charcoal in 50 mM KH2PO4, pH 2.3). After pelleting the charcoal by centrifugation, [32P]phosphate was measured in the supernatant.

Activation and deactivation of phosphorylated vs. nonphosphorylated PDE5
Aliquots of cGKI-depleted or cGKI-containing platelet supernatant were treated with alkaline phosphatase and Rp-8-Brc-cAMPS as described in the previous paragraph. An aliquot was withdrawn and diluted 1,000-fold into ice-cold dilution buffer for determination of the basal PDE activity. cGMP preincubation was started by addition of cGMP and MgCl2 to the diluted supernatant (final concentration 100 μM and 10 mM, respectively). After 1 min of preincubation at 37°C, an aliquot was withdrawn and diluted into ice-cold dilution buffer for determination of maximal PDE activity. Another aliquot was diluted 250-fold into dilution buffer warmed to 37°C, and aliquots were withdrawn and put on ice at the indicated times. 50 μl of the samples were assayed for PDE activity at 1 μM cGMP as substrate for 5 min in a total volume of 100 μl.

Activation and deactivation of PDE5 in diluted cGKI-depleted platelet supernatants
Platelets supernatant prepared as described above was diluted 20-fold into buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM triethanolamine/HCl, pH 7.4, and was treated with 55 U/ml of alkaline phosphatase for 5 min at 37°C, followed by the addition of 100 μM Rp-8-Brc-cAMPS for another minute. Subsequently, an aliquot was withdrawn into ice-cold dilution buffer for determination of basal PDE activity. cGMP preincubation was started by the addition of cGMP and MgCl2 to the diluted supernatant (final concentration 100 μM and 10 mM, respectively). After 1 min of preincubation at 37°C, an aliquot was withdrawn and diluted into ice-cold dilution buffer for determination of maximal PDE activity. Another aliquot was diluted 250-fold into dilution buffer warmed to 37°C, and aliquots were withdrawn and put on ice at the indicated times. 50 μl of the samples were assayed for PDE activity at 1 μM cGMP as substrate for 5 min in a total volume of 100 μl.

Determination of cGMP degradation during preincubation of supernatants
Aliquots of dilution buffer for determination of the basal PDE activity. cGMP preincubation was started by addition of cGMP and MgCl2 (final concentration 100 μM or 500 μM cGMP and 10 mM MgCl2) in the presence of 500 μM ATP (cGKI-containing supernatant), or in the absence of ATP (cGKI-depleted supernatant) for 5 min at 37°C. An aliquot was withdrawn and diluted 1,000-fold into ice-cold dilution buffer for determination of maximal PDE activity. Another aliquot was diluted 1,000-fold into prewarmed (37°C) dilution buffer and aliquots were withdrawn and put on ice at the indicated times. 30 μl of the samples were assayed for PDE activity at 1 μM cGMP as substrate for 5 min in a volume of 100 μl.

Determination of cGMP levels in human platelets
Platelets were freshly prepared as described above; the NO incubations and the subsequent determination of cGMP using an RIA were performed as described previously (Mullershausen et al., 2001).

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