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Biochemical Pharmacology 67 (2004) 1579–1585
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Native human nitric oxide sensitive guanylyl cyclase: purification and characterization

Markus Koglin, Sönke Behrends*

Institut für Experimentelle und Klinische Pharmakologie, Universität Hamburg, Martinistrasse 52, Hamburg D-20251, Germany

Received 21 August 2003; accepted 5 January 2004

Abstract

The only published report of the purification of native human soluble guanylyl cyclase (sGC) used placenta as starting material. This enzyme preparation showed low fold-activation by NO and a maximal absorption of the prosthetic heme-group at 417 nm indicative of a prosthetic heme-group in a hexa-coordinate state. These data are in contrast to what has subsequently been found for the recombinant human enzymes. Apart from this placental enzyme preparation, a native functional human NO-sensitive sGC has not been successfully purified. The aim of the current study was to purify and characterize native human sGC from another source, to see whether the discrepancies between native and recombinant sGC seen for placenta are a general phenomenon. We chose human platelets as starting material since the properties of this enzyme are directly relevant for the development of innovative antiplatelet and antianginal drugs. Our results indicate that the native platelet enzyme exists as a highly NO-sensitive, heterodimeric enzyme with an α_1 and β_1 subunit. In contrast to the native human placental enzyme and in accordance with the human recombinant enzymes, the native human platelet enzyme contains a ferrous, penta-coordinate heme group. To our knowledge this is the first report of the successful purification and characterization of the native human nitric oxide sensitive α_1/β_1 isoform of sGC which is widely expressed in the cardiovascular system and is an important target of innovative drugs.

Keywords: Guanylyl cyclase; Cyclic GMP; Nitric oxide; Platelets

Nitric oxide (NO)-sensitive or soluble guanylyl cyclase (sGC) is the target of classical NO-releasing drugs like glyceryl trinitrate that have been successfully used as remedy for angina pectoris for more than hundred years [1]. Recently, novel drugs have been developed at major pharmaceutical companies (Bayer, Aventis and Abbott) that activate the enzyme independent of the release of NO [2–4]. Beyond their possible use in angina pectoris, these drugs are in contrast to glyceryl trinitrate potent antiplatelet drugs (for review see [5]).

Several groups have purified native, NO sensitive sGC from bovine or rat lung, rat brain and rat liver and characterized the enzyme as a penta-coordinate, ferrous hemoprotein consisting of two subunits α_1 and β_1 (for review see [6]). After the cloning of these subunits in a number of species, these results have been confirmed using enzyme overexpressed in the baculovirus/Sf9-system. Recently, the human recombinant α_1/β_1 isoform has been overexpressed in the baculovirus/Sf9-system, purified and characterized as a penta-coordinate, ferrous hemoprotein [7–9]. Two other subunit cDNAs have been cloned by homology screening: the β_2 subunit from rat kidney and the α_2 subunit from human fetal brain [10,11]. Co-expression of the α_1/β_1 and α_2/β_1 cDNAs yielded NO-sensitive enzymes in expression systems [11], and the α_2/β_1 heterodimeric enzyme has been demonstrated on the protein level in human placenta by co-precipitation experiments and also characterized as a penta-coordinate, ferrous hemoprotein [12]. We could recently isolate a β_2 cDNA variant from rat kidney that shows NO-sensitive enzyme activity after expression in Sf9 or HEK-293 cells in the absence of a second subunit, most likely as β_2/β_2 homodimer [13]. Cloning of the human ortholog of the rat β_2 subunit revealed a frame-shift in the amino-terminal, potential heme-binding region in the general population, indicating that this subunit does probably not act as NO-receptor in man [14].

* Corresponding author. Tel.: +49-40-42803-2055; fax: +49-40-42803-4876.
E-mail address: behrends@uke.uni-hamburg.de (S. Behrends).

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doi:10.1016/j.bcp.2004.01.007

The only published report of the purification of native human sGC used placenta as starting material [15]. This enzyme preparation showed low fold-activation by NO in comparison to the bovine lung enzyme. In addition, spectral analysis showed a maximal absorption of the prosthetic heme-group at 417 nm indicative of a prosthetic heme-group in a hexa-coordinate state. These data are in contrast to what has subsequently been found for the recombinant human enzymes [7–9]. Apart from this placental enzyme preparation, a native functional human NO-sensitive sGC has not been successfully purified.

The aim of the current study was to purify and characterize native human sGC from another source, to see whether the discrepancies between native and recombinant sGC seen for placenta are a general phenomenon. We chose human platelets as starting material since the properties of this enzyme are directly relevant for the development of innovative antiplatelet and antianginal drugs [5,16].

1. Materials and methods

1.1. Materials

Human platelet concentrates were obtained from the local blood bank of the University Clinic Hamburg-Eppendorf. Platelet concentrates were approved by the Federal Institute for Drugs and Medical Devices (BFARM: 10574a/96-1-4) and underlie strict quality control. Concentrates produced in excess of the demands for clinic routine were processed immediately after receipt from the local blood bank. The gel filtration calibration kit was from Amersham Pharmacia Biotech (Piscataway, USA). 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO) and all other chemicals were obtained from Sigma (St. Louis, USA) in the highest grade of purity.

1.2. Purification of sGC

Approximately 100 g centrifuged platelets (wet weight) were thawed (storage at −70 °C) in a water bath at room temperature for 8 min. All following steps were performed at 4 °C or on ice. The cell pellet was resuspended in 240 ml 50 mM TEA/HCl pH 8.0 containing 10 mM DTT, 1 mM benzamidine, 10 μg/ml PMSF, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 900 μl protease inhibitor mix (Sigma). The solution was homogenized with a cell disruption bomb (Parr, Moline, USA) at 60 bar for 45 min. The homogenate was then centrifuged at 40,000 × *g* for 30 min, and 240 ml supernatant was collected. All chromatographic steps were performed on a FPLC system (Amersham Pharmacia Biotech). The protease inhibitor benzamidine (1 mM), DTT (10 mM) and PMSF (10 μg/ml) were used in all chromatographic steps. The supernatant was immediately applied to a Q-Sepharose column (20 ml volume) at 2 ml/min. Ion exchange buffer A contained 50 mM TEA/HCl pH 8.0. Ion exchange buffer B contained 5 mM potassium phosphate (pH 7.2). Ion exchange buffer C was prepared by adding 1 M NaCl to buffer B. The column was washed at 3 ml/min with buffer A, buffer B and 8% buffer C until OD_{280} was stable. A linear gradient from 8% C to 30% C for 828 ml was used to elute sGC. The sGC containing fractions were pooled by determining sGC activity at basal and NO-stimulated conditions after each column. The pooled fractions (104 ml) were diluted with 936 ml 5 mM potassium phosphate (pH 7.2) and applied to a Blue-Sepharose column (Amersham Pharmacia Biotech, 5 ml volume) at 1.0 ml/min. Blue-Sepharose buffer A containing 5 mM potassium phosphate (pH 7.2) and Blue-Sepharose buffer B was prepared by adding 1 M NaCl buffer A. The column was then washed with 10% buffer B until the OD_{280} was stable. The enzyme was eluted with a linear gradient running from 10% B to 100% B for 120 ml. The sGC containing fractions (60 ml) were diluted with 60 ml 5 mM potassium phosphate (pH 7.2) and applied immediately to a ceramic Hydroxyapatite column (BioRad, Hercules, USA, 5 ml volume) at 1.5 ml/min. Hydroxyapatite buffer A containing 5 mM potassium phosphate (pH 7.2) and Hydroxyapatite buffer B containing 400 mM potassium phosphate (pH 6.6). The column was then washed with buffer A until the OD_{280} was stable. The enzyme was eluted with a linear gradient running from 0% B to 60% B for 90 ml. The sGC containing fractions (24 ml) were again pooled and concentrated in centrifugal devices with a 50-kDa cut-off (Millipore, Bedford, USA) to approximately 1.5 ml. The enzyme was then loaded on a Superdex 200 column (Amersham Pharmacia Biotech, 60 cm × 2.6 cm) and eluted overnight with 50 mM TEA/HCl pH 8.0 containing 250 mM NaCl at 0.15 ml/min. Fractions with the highest sGC activity were pooled and concentrated as described above to a final volume of approximately 300 μl. For spectroscopic measurements 100 μl of purified enzyme were used. Purified enzyme was diluted with 50 mM TEA/HCl pH 8.0 containing 250 mM NaCl and stored with 10% (v/v) glycerol at −80 °C.

1.3. Determination of protein concentration and guanylyl cyclase activity assay

Protein concentrations were determined by the method of Bradford using bovine plasma gamma globulin (Protein Assay Standard I, BioRad) as standard. The protein contents of purified fractions were confirmed by calculating the protein concentration from optical densities using the formula [17]:

$$c = 1.55(A_{280} - A_{320}) - 1.76(A_{260} - A_{320})$$

Guanylyl cyclase activity of purified protein (20 ng protein per assay tube) was determined by incubation for 10 min at 37 °C in the presence of 1 mM cGMP, 0.5 mM [^{32}P]GTP (about 0.2 μCi), 3 mM $MgCl_2$,

50 mM TEA/HCl, pH 7.4, 0.25 g/l creatine kinase, 5 mM creatine phosphate and 1 mM 3-isobutyl-1-methylxanthine in a total volume of 0.1 ml as described by Schultz and Böhme [18]. Reactions were started by the addition of protein and incubation at 37 °C. All experiments were stopped by $ZnCO_3$ precipitation, and purification of the enzyme-formed cGMP was performed as described [18]. Basal enzyme activity measurements were performed in the absence of NO. NO-stimulated measurements were performed in the presence of the NO-donor DEA/NO.

1.4. SDS–PAGE and immunoblotting

Generation of the α_1-1200 and the β_1-89 antibodies were described previously [9]. For monitoring the purity of enzyme preparations and for the determination of apparent molecular masses of the purified enzyme, SDS–PAGE was performed in 10% slab gels and were stained with Coomassie blue G-250. For immunoblotting protein fractions were run on SDS–PAGE gels and transferred electrophoretically to a nitrocellulose membrane. The membrane was reversibly stained with Ponceau S and unspecific binding sites were saturated by immersing the membrane for 1 h in TBST-buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk. The membranes were incubated for 1.5 h in TBST-buffer containing α_1-1200 and β_1-89 in a 1:2000 dilution and 0.5% dry milk. Negative control reactions were run in additional presence of synthetic peptides used for immunization in different combinations (5 μg/ml). The membranes were washed three times for 10 min with TBST and subsequently incubated for 1 h with horseradish peroxidase labeled anti-rabbit-IgG antibodies (diluted 1:4000, Sigma). After three washes with TBST the membranes were processed with the ECL Western blotting detection system according to the recommendations of the manufacturer (Amersham Pharmacia Biotech).

1.5. Statistical analysis

All results were controlled for their statistical significance by student *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

2. Results

Starting with 240 ml of human platelet cytosol from approximately 100 g (wet weight) platelet pellet, we obtained 0.35 mg protein with a specific activity of 34.1 nmol cGMP/min per mg in absence of DEA/NO and 529-fold purification with a recovery of 6.6% (Table 1). Specific enzyme activity increased to 2958 nmol cGMP/min per mg in the presence of 100 μM DEA/NO and yielded a 1105-fold purification with a recovery of 13.7% (Table 1). After each chromatographic step sGC activity was measured under basal conditions and in the presence of 100 μM DEA/NO in the recovered fractions. Only fractions with highest cGMP production were used in subsequent purification steps (Fig. 1). Frozen aliquots of pooled fractions after each purification step were used to monitor the purification process. Representative results of each purification step are summarized in Table 1.

A Coomassie blue stained SDS–PAGE analysis is shown in Fig. 2 (left panel). Two major signals with molecular masses of 80 and 72 kDa were identified, which is close to the estimated molecular masses of sGC α_1- and β_1-subunit (77.5 and 70.5 kDa, SWISS PROT data bank). Two very faint bands with a molecular mass of approximately 50 and 100 kDa were also apparent. To control the identity of the bands on the Coomassie blue stained SDS–PAGE gel, we eluted the bands from the gel, did a proteolytic digest with trypsin and analyzed fragments by mass spectrometry. The 50 kDa band was identified as adaptor-related protein complex 1 (GenBank accession number: NP_115882). The 100 kDa band was identified as complement component 3 precursor (GenBank accession number: NP_000055). The major bands were identified as the human α_1 subunit and β_1 subunit of sGC formerly designated α_3 (GenBank accession number: NP_000847) and β_3 (GenBank accession number: NP_000848). In the Western immunoblot analysis (Fig. 2, right panel), we used antibodies directed against carboxy-terminal sequences of the α_1- and β_1-subunit and compared the platelet enzyme preparation to the purified human recombinant α_1/β_1 sGC expressed in the Sf9/baculovirus system that has recently been characterized by our group [9]. In each lane only a single band was observed that was displaced by the

Table 1
Purification of sGC from platelets

Step	Protein (mg)	Volume (ml)	Guanylyl cyclase activity (nmol cGMP/min per mg)		Purification (*x*-fold)	
			Basal	DEA/NO	Basal	DEA/NO
Cytosol	2835	240	0.064	2.7	1	1
Q-Sepharose	135	104	4.4	196	68	73
Blue-Sepharose	15.7	60	6.7	314	103	117
Hydroxyapatite	4.0	24	23	351	358	131
Size exclusion	0.35	1.6	34.1	2958	529	1105

Activities were measured in presence or absence of 100 μM DEA/NO and with 3 mM magnesium as cofactor. The results are representative of three similar independent experiments.

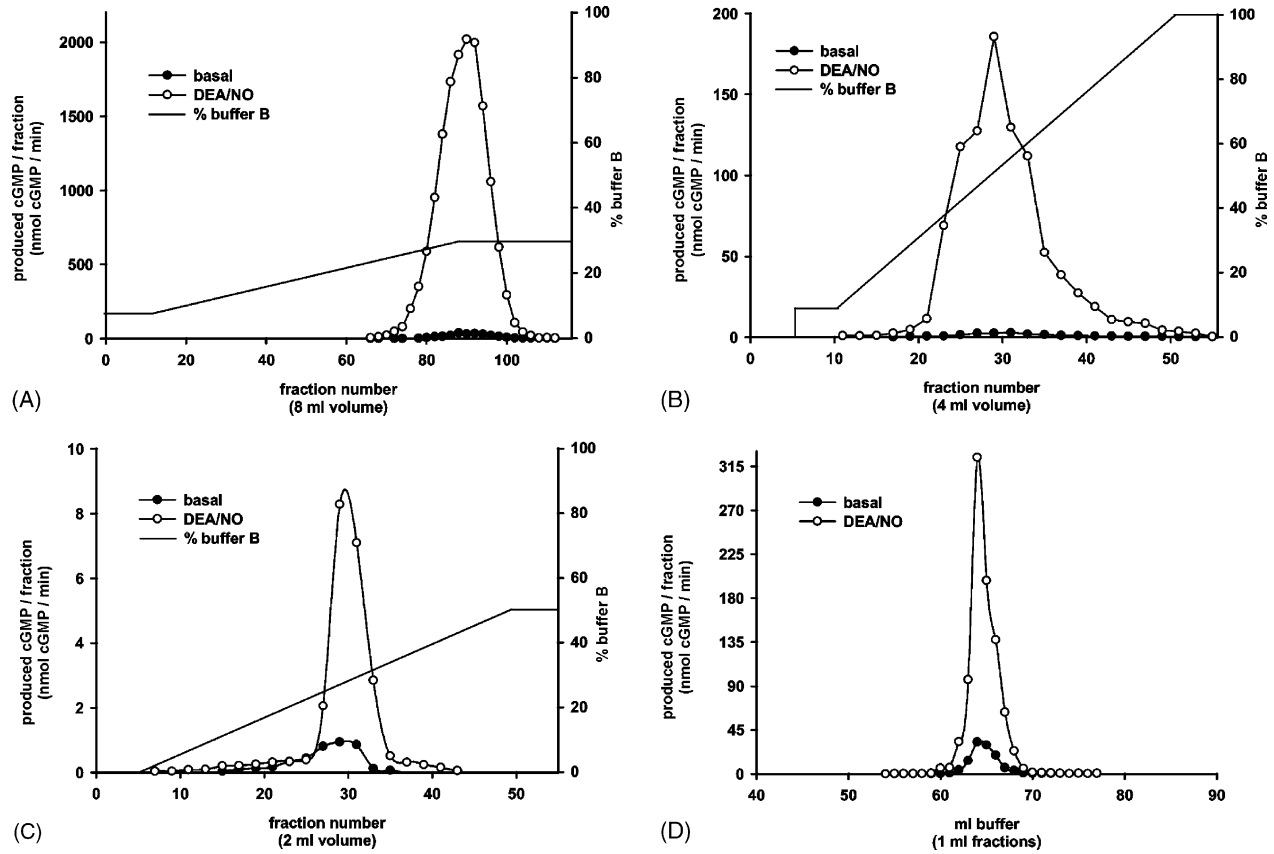

Fig. 1. Characterization of purification of sGC from human platelets. Elution profiles of sGC are shown for Q-Sepharose (A), Blue-Sepharose (B), Hydroxyapatite (C), and Gel filtration-column (D). Guanylyl cyclase activity was measured under basal conditions (closed circles) or in the presence of 100 μM DEA/NO (open circles) and is shown as produced cGMP/min in each fraction. The following fractions were pooled and used in the next purification step: Q-Sepharose (fractions 84–95); Blue-Sepharose (fractions 23–37); Hydroxyapatite (fractions 25–36); Gel filtration (fractions 63–66).

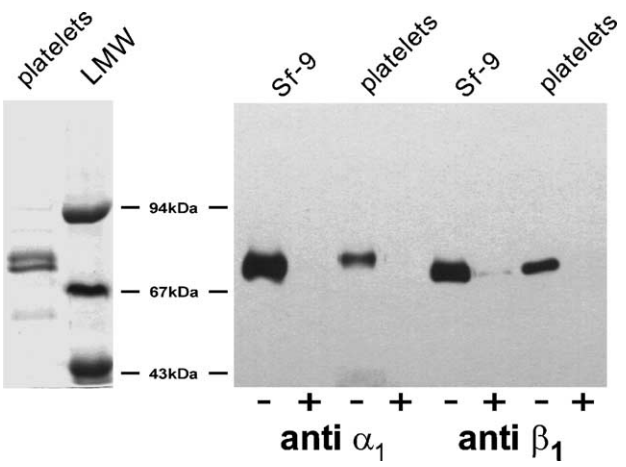

Fig. 2. SDS–PAGE analysis of purified sGC and characterization of the subunits by Western-blot analysis. One microgram of purified enzyme was electrophoresed by 10% SDS–PAGE and stained with Coomassie blue (left panel). For Western-blot analysis an α_1 (AK 1200) and a β_1 (AK89) antibody were used in a 1:2000 dilution. One microgram of purified enzyme obtained from platelets and Sf-9 overexpression system (9) was electrophoresed by 10% SDS–PAGE and transferred to nitrocellulose membranes and incubated with the respective antibodies. The respective subunit of sGC was determined by blocking antibody signals with 5 μg/ml of the respective peptides (right panel). In the middle a low molecular weight standard (LMW) is shown.

respective peptides used for raising the antibodies. The molecular masses of the subunits in recombinant human sGC overexpressed in the baculovirus Sf9-system appeared identical to the subunits of sGC purified from human platelets.

Gel filtration of platelet sGC was performed together with a number of standard proteins (Albumin 67 kDa, Aldolase 158 kDa, Catalase 232 kDa, Ferritin 440 kDa, Thyroglobuline 669 kDa) to determine the molecular mass of the native enzyme complex (Fig. 3). The native sGC from human platelets showed an apparent molecular mass of 160 kDa which is close to the theoretical calculated value for the dimeric α_1/β_1 enzyme complex (148 kDa). Higher ordered complexes with a 1:1 stochiometry of α_1 to β_1 like a tetrameric enzyme complex with a theoretical calculated molecular mass of 296 kDa were not apparent (Fig. 3).

Spectral analysis of the purified sGC complex from human platelets revealed absorption maxima at 280 and 430 nm (Fig. 4). While the peak at 280 nm is due to the presence of aromatic amino acids in the protein, the peak at 430 nm is characteristic of the prosthetic heme group in sGC (Soret band). The Soret band shifted from 430 to 398 nm in the presence of the NO-donor DEA/NO

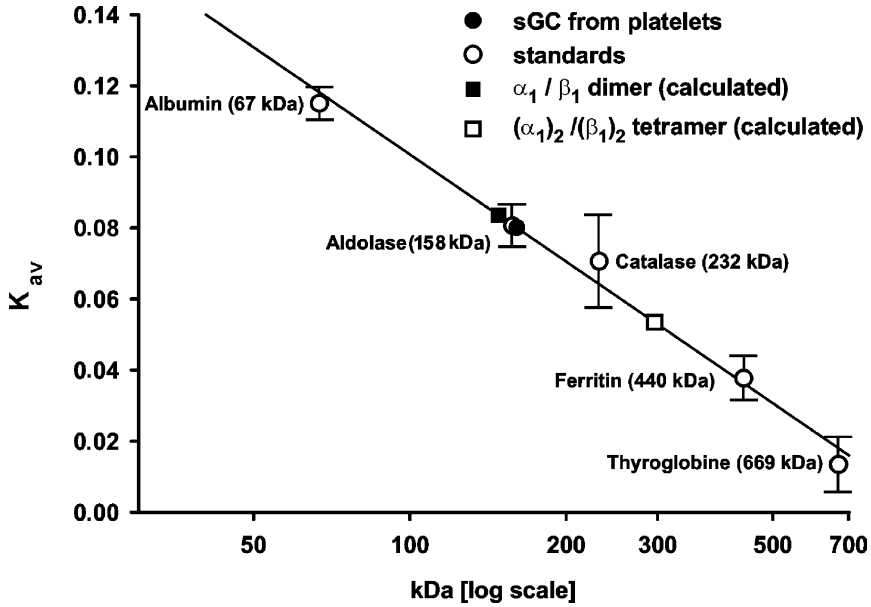

Fig. 3. Estimation of the molecular mass of native purified platelet sGC. Calibration of the gel filtration column was performed with a mixture of different proteins (see figure, including error bars) according to the recommendations of the manufacturer. The column void volume was estimated with Blue Dextran 2000. Molecular masses for dimeric and tetrameric sGC were predicted from their respective cDNA species.

(100 μM). This indicates binding of NO to the prosthetic heme group of sGC and formation of a penta-coordinated nitrosyl-heme complex.

To examine the kinetic properties of purified sGC from human platelets, cGMP formation was determined in the presence of increasing GTP concentrations (Fig. 5). A non-linear regression was performed using the Michaelis–Henri equation. The non-linear plot of the data revealed a K_M-value of 194 ± 15 μM GTP in the absence of NO (Fig. 5A). The K_M-value was significantly reduced to 57 ± 4 μM GTP ($P < 0.001$) in the presence of 100 μM DEA/NO (Fig. 5B). The V_{max}-value of 57 ± 1 nmol cGMP/min per mg at basal conditions increased to 2555 ± 43 nmol cGMP/min per mg in the presence of DEA/NO ($P < 0.001$). Concentration response experiments using

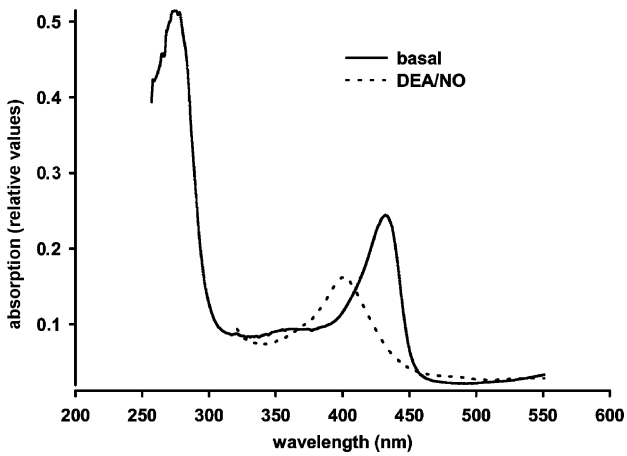

Fig. 4. Spectroscopic analysis of purified guanylyl cyclase enzyme complex. Heme spectra of sGC under basal conditions (solid line) and in the presence of 100 μM DEA/NO (dotted line).

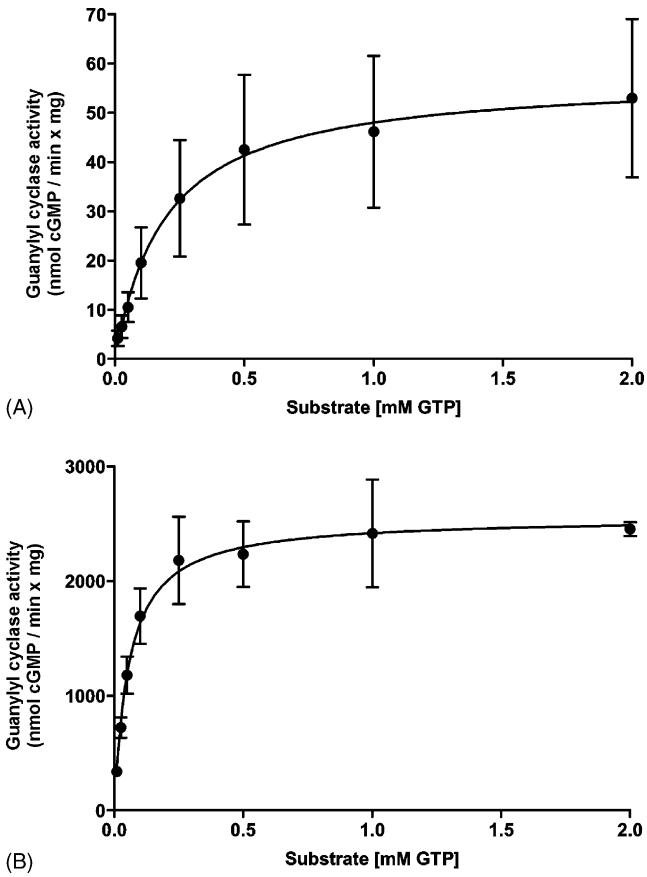

Fig. 5. Non-linear regression for substrate dependency of purified sGC. Substrate dependency was measured in a range from 0.01 to 2 mM GTP at basal (A) or NO-stimulated (B, 100 μM DEA/NO) conditions in the presence of 3 mM Mg^{2+}. The non-linear regression plot was performed using the Michaelis–Henri equation. Data represent means of three independent experiments performed in duplicate (±S.E.M.).

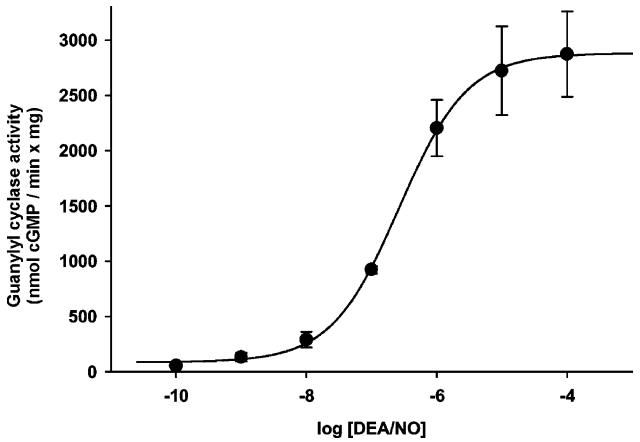

Fig. 6. Concentration-dependent effect of DEA/NO on guanylyl cyclase activity purified from platelets. Dose–response curve was measured in a range of 0.1 nM–100 μM DEA/NO. Substrate concentration and additionals within the reaction mixture are mentioned in Section 1. All values show the result of three independent experiments performed in duplicate.

DEA/NO led to a concentration dependent activation of platelet sGC with an 87-fold increase in the catalytic activity (k_{cat}) in presence of 100 μM DEA/NO and an EC_{50}-value of 255 ± 58 nM DEA/NO (Fig. 6).

3. Discussion

The only previous report of a successful purification of native functional human NO-sensitive sGC used human placenta as starting material [15]. This native sGC from human placenta was activated five-fold by NO (from 25 to 128 nmol cGMP/min per mg) by NO while enzyme purified from bovine lung that was used as reference was activated 25-fold (from 20 to 495 nmol cGMP/min per mg) under the same conditions [15]. The native platelet enzyme in the current study was activated 87-fold by NO. In the spectral analysis sGC purified from human placenta showed an absorption maximum in the Soret region at 417 nm [15]. This indicates that the iron of the heme group in placental sGC is in a hexa-coordinate state [15]. The native human platelet enzyme showed absorbance spectra of the heme group with a maximum of the Soret band at 430 nm indicative of a penta-coordinated ferrous heme with a histidine as the axial ligand. The Soret band shifted to 398 nm in the presence of NO due to the formation of a penta-coordinated nitrosyl-heme complex. Characterization of the heme microenvironment by spectral analysis of the α_2/β_1 isoform and the recombinant α_1/β_1 isoform are consistent with our current findings for native human platelet sGC [7–9,12]. Thus the microenvironment of the heme group of native placental sGC seems to differ considerably from that of the native human platelet enzyme and the recombinant human forms analyzed thus far. The respective subunits of native human placental sGC showed apparent molecular masses of 70 and 88 kDa. While the 70 kDa subunit corresponds to the β_1 subunit of the native human platelet enzyme, the 88 kDa band in the placental enzyme differs from the platelet α_1 subunit at 80 kDa and probably represents the α_2 subunit that is known to be expressed in placental tissue [12,19]. This would indicate that there is a difference with respect to the prosthetic heme group between the recombinant α_2/β_1 isoform and the native α_2/β_1 isoform.

The native platelet enzyme isolated in the current study is made up of two subunits that display an identical molecular mass as the recombinant human α_1 and β_1 subunits and are recognized by the respective specific antibodies directed against α_1 and β_1 in the Western blot. The specific activity of the native platelet enzyme in the presence of NO was in the same range as the human recombinant α_1/β_1 enzyme with a hexa-histidine tag (3 μmol/min per mg, [7]). The native platelet enzyme was activated by NO 87-fold. This is high in comparison to the native human enzyme preparation from placenta which was activated only five-fold by NO [15] and within the range of what has been published for the human recombinant α_1/β_1 isoform enzyme without modification purified from High Five- (10-fold [8]) or Sf9-insect cells (242-fold, [9]).

In a previous report a purification of soluble guanylyl cyclase from human platelets has been described [20]. However, in this very short paper potential activation of the enzyme by nitric oxide was not reported. The enzyme preparation was only tested with respect to basal activity in the presence of Mn^{2+} at a constant substrate concentration of 0.5 mM GTP. The paper also does not contain any evidence that the purified enzyme contained heme. Although the identity of the subunits was not characterized the molecular weights are consistent with what we report in the current paper for the α_1 and β_1 subunits. Given that the very easy experiment of activation of the enzyme by a NO-donor was not reported, it is likely that a non-heme containing NO-insensitive apoprotein was the result of the purification protocol described in Ref. [20].

Purification of native sGC from tissue of animals has so far resulted in preparations with a 1:1 stochiometry of the α_1 and β_1 subunit [6] and this is consistent with what we find for native human sGC from platelets in the current study. Whether native human sGC exists as dimer or higher-ordered species (e.g. tetramer), has so far not been addressed. Also it has very recently been described that the β_1 subunit of sGC can interact with heat shock protein 90 in different cell types [21] and heat shock protein 90 is expressed in human platelets [22]. We observed two very faint bands at approximately 100 and 50 kDa in the human platelet preparation. By mass spectrometry they were assigned to proteins of the complement system and clathrin coated vesicles which are unrelated to sGC. Gel filtration experiments in the current study gave an estimated molecular mass of native human platelet sGC of about 160 kDa which is in good accordance with the calculated value of the α_1/β_1 heterodimer. This indicates that native human

platelet sGC exists as heterodimer and that it does not bind stochiometric amounts of yet another protein.

Since we have so far not found a significant difference between the native human α_1/β_1 heterodimeric enzyme from platelets and the respective human recombinant enzyme expressed in Sf9-cells, it is reasonable to use the latter in drug development. However, we believe that it is crucial to check the activity of drugs at later stages of pre-clinical drug development with the native human enzyme preparation as described in the current study because differences might nevertheless exist. This relates in particular to possible posttranslational modifications which could conceivably block binding of a novel substance or alter its affinity. For example quite novel data which have so far only described in abstract form, indicate that the α_1/β_1 heterodimeric form of nitric oxide sensitive guanylyl cyclase is in part regulated by tyrosine phosphorylation [23].

In summary, we purified and characterized native human NO-sensitive sGC from platelets. Our results indicate that the native platelet enzyme exists as a highly NO-sensitive, heterodimeric enzyme with an α_1 and β_1 subunit. In contrast to the native human placental enzyme and in accordance with the human recombinant enzymes, the native human platelet enzyme contains a ferrous, pentacoordinate heme group. To our knowledge this is the first report of the successful purification and characterization of the native human NO-sensitive α_1/β_1 isoform of sGC which is widely expressed in the cardiovascular system and is an important target of innovative drugs [5,16].

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