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Nucleotidyl Cyclase Activity of Soluble Guanylyl Cyclase $\alpha_1\beta_1$

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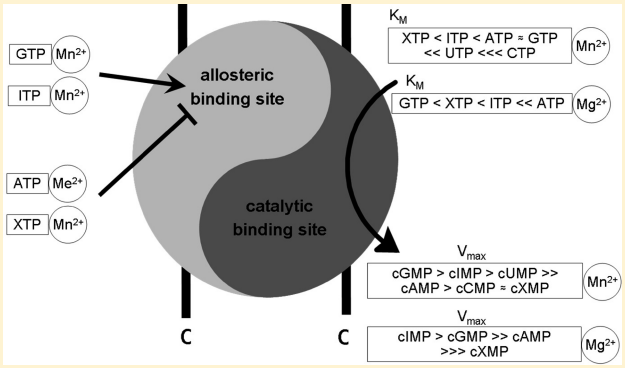
Supporting Information

ABSTRACT: Soluble guanylyl cyclase (sGC) regulates several important physiological processes by converting GTP into the second-messenger cGMP. sGC has several structural and functional properties in common with adenylyl cyclases (ACs). Recently, we reported that membranous ACs and sGC are potently inhibited by 2′,3′-*O*-(2,4,6-trinitrophenyl)-substituted purine and pyrimidine nucleoside 5′-triphosphates. Using a highly sensitive high-performance liquid chromatography–tandem mass spectrometry method, we report that highly purified recombinant sGC of rat possesses nucleotidyl cyclase activity. As opposed to GTP, ITP, XTP and ATP, the pyrimidine nucleotides UTP and CTP were found to be sGC substrates in the presence of Mn^{2+}. When Mg^{2+} is used, sGC generates cGMP, cAMP, cIMP, and cXMP. In conclusion, soluble "guanylyl" cyclase possesses much broader substrate specificity than previously assumed. Our data have important implications for cyclic nucleotide-mediated signal transduction.



Guanylyl cyclases (GCs) make up a family of enzymes that catalyze the biosynthesis of guanosine 3′,5′-cyclic monophosphate (cGMP) from guanosine 5′-triphosphate (GTP). The family consists of two isoforms, the soluble isoform that is expressed in cytoplasm and the particulate or membrane-bound form.[1−3] The soluble GC (sGC) is a heterodimer consisting of an α-subunit and a β-subunit containing a heme as a prosthetic group.[4] Two sGC isoforms have been identified. The best-studied sGC isoform is termed $\alpha_1\beta_1$ and is expressed in most tissues.[4] Further subunits have also been cloned, i.e., α_2, α_{2i} (a splice variant of α_2), β_2, and β_3. However, only $\alpha_2\beta_1$ has been detected in vivo. The $\alpha_2\beta_1$ isoform is expressed in brain and in fetal tissue.[4] In vitro studies with $\alpha_2\beta_1$ showed very similar NO and carbon monoxide binding characteristics as well as inhibition by [1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) and stimulation by YC-1 {5-[1-(phenylmethyl)-1*H*-indazol-3-yl]-2-furanmethanol} compared to $\alpha_1\beta_1$, although the sequences of α_1 and α_2 are only 27% identical.[5] The discovery of other possible sGC subunit combinations in vivo remains elusive.

Nitric oxide (NO) activates sGC up to 100−400-fold.[4] Stimulation of sGC leads to accumulation of cGMP. cGMP is a second messenger regulating cGMP-dependent protein kinases, phosphodiesterases, and ion channels. cGMP signaling plays an important role in many (patho)physiological processes like smooth muscle relaxation and neurotransmission.[6] Heme of sGC can be oxidized by reactive oxygen species or by ODQ, which is followed by a loss of NO sensitivity.[6−8]

Adenylyl cyclases (ACs) are structurally and functionally related to GCs. Recently, we demonstrated that membranous ACs (mACs) are very potently inhibited by 2′,3′-*O*-(2,4,6-trinitrophenyl) nucleotides (TNP-nucleotides) and 2′(3′)-*O*-(*N*-methylanthraniloyl) nucleotides (MANT-nucleotides).[9−11] For GCs, in 1969, supernatant fractions of rat lung were shown to be inhibited by adenosine 5′-triphosphate (ATP), inosine 5′-triphosphate (ITP), cytidine 5′-triphosphate (CTP), and uridine 5′-triphosphate (UTP),[12] and some years later, sGC purified from rat brain was shown to be inhibited by CTP, UTP, and ATP in a competitive manner.[13] Furthermore, sGC produces adenosine 3′,5′-cyclic monophosphate (cAMP)[9,14,15] and bona fide uridine 3′,5′-cyclic monophosphate (cUMP)[9] as assessed by a classical radiometric nucleotidyl cyclase (NC) assay. ATP is a mixed-type inhibitor of sGC, indicating that sGC contains an allosteric nucleotide binding site.[15] Moreover, soluble fractions of rat lung generate inosine 3′,5′-cyclic monophosphate (cIMP) besides cGMP.[16] Finally, the biosynthesis of cGMP by sGC is also potently inhibited by MANT- and TNP-substituted purine and pyrimidine nucleotides.[9,10]

However, the assumed broader NC activity of sGC was always a matter of controversy because of crude enzyme preparations, impure analytical standards, or cross reactivity of antibodies.[12,16−22] In the present study, for the first time, comprehensive kinetic parameters are obtained for synthesis of seven cyclic purine and pyrimidine nucleotides by highly purified recombinant rat sGC isoform $\alpha_1\beta_1$. Because not all NTPs are commercially available as radiolabeled analogues and

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Table 1. Parameters for the Detection and Identification of cNMPs and MS Standard Tenofovir[a]

	cAMP	cCMP	cGMP	cIMP	cTMP	cUMP	cXMP	tenofovir
$[M + H]^+$ (m/z)	330.1	306.1	346.1	331.1	305.1	307.0	347.1	288.2
quantifier (m/z)	136.2	112.1	152.1	137.2	127.1	113.1	153.1	176.2
qualifier (m/z)	119.1	95.0	135.2	110.1	81.0	97.0	136.2	270.2
quantifier/qualifier ratio	1:10	1:6.8	1:3.2	1:5.3	1:3.7	1:1.1	1:3.1	1:1.1
retention time (min)	4.4	2.9	3.3	3.4	3.7	3.1	2.7	3.6

[a]Protonated molecular masses ($[M + H]^+$), HPLC retention times, quantifier and qualifier MS/MS fragments, and quantifier:qualifier ratios are given.

structure-based identification of products has not yet been performed, we have developed an analytical method based on high-performance liquid chromatography and tandem mass spectrometry (HPLC−MS/MS) for simultaneous detection and quantitation of seven nucleoside 3′,5′-cyclic monophosphates (cNMPs): adenosine 3′,5′-cyclic monophosphate (cAMP), cytidine 3′,5′-cyclic monophosphate (cCMP), guanosine 3′,5′-cyclic monophosphate (cGMP), inosine 3′,5′-cyclic monophosphate (cIMP), thymidine 3′,5′-cyclic monophosphate (cTMP), uridine 3′,5′-cyclic monophosphate (cUMP), and xanthosine 3′,5′-cyclic monophosphate (cXMP).

MATERIALS AND METHODS

NTPs of adenine (2Na·ATP, ≥99%), uracil (3Na·UTP, ≥96%), hypoxanthine (3Na·ITP, 95−97%), and guanine (GTP, ≥95%) as well as triethylamine (TEA), ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), Na·cAMP, magnesium chloride, dithiothreitol (DTT), bovine serum albumin (BSA), sodium nitroprusside (SNP), ODQ, dimethyl sulfoxide (DMSO), and sodium acetate were purchased from Sigma-Aldrich (Seelze, Germany). Cytidine 5′-triphosphate (CTP, >99%), xanthosine 5′-triphosphate (XTP, >95%), and thymidine 5′-triphosphate (dTTP, >95%) were obtained from Jena Bioscience (Jena, Germany). Tris(hydroxylmethyl)-aminomethane hydrochloride (Tris-HCl) was purchased from Merck (Darmstadt, Germany). cGMP, cUMP, cIMP, cCMP, cXMP, and cTMP (cNMP·Na) were supplied by Biolog (Bremen, Germany). Manganese chloride tetrahydrate, hydrochloric acid, and ammonium acetate were purchased from Fluka (Buchs, Germany). Acetonitrile, methanol, and water were supplied by Baker (Deventer, The Netherlands), and acetic acid was purchased from Honeywell/Riedel-de Haën (Seelze, Germany). [α-^{32}P]GTP (3000 Ci/mmol) was obtained from Hartmann Analytic (Braunschweig, Germany). Aluminum oxide N Super 1 was purchased from MP Biomedicals (Eschwege, Germany). Tenofovir was obtained through the National Institute of Health AIDS Research and Reference Program, Division of AIDS (catalog no. 10199) (Bethesda, MD). sGC $\alpha_1\beta_1$ was purified as described in ref 23.

Nonradioactive NC Assay of sGC $\alpha_1\beta_1$. Directly before each experiment, the sGC activator SNP was dissolved in a light-protected brown tube in 100 mM sodium acetate (pH 5.0). NC activities were analyzed at 37 °C in a reaction buffer containing 50 mM triethanolamine (TEA) (pH 7.5), 100 μM EGTA, 3 mM free Mn^{2+} or Mg^{2+}, 1 mM DTT, 1 mg/mL BSA, and 100 μM SNP or 100 μM ODQ [0.3% (v/v) DMSO] and SNP in a final volume of 50 μL. The NTP/Me^{2+} concentration ranged from 2 to 7500 μM. Depending on the analyzed NTP, reaction was initiated by the addition of 0.1−50 ng of purified enzyme. After 5−60 min, assays were stopped when the samples were heated at 95 °C for 10 min. After cooling, mixtures were diluted with 50 μL of a 97:3 (v/v) water/methanol mixture containing 50 mM ammonium acetate, 0.1% (v/v) acetic acid, and 100 ng/mL tenofovir. Denatured protein was precipitated by centrifugation for 10 min at 20000*g*.

HPLC−MS/MS Quantitation of NC Assays. cNMPs were separated using a Series 200 HPLC system (Perkin-Elmer, Norwalk, CT) equipped with a binary pump system, a degasser, and a temperature-controlled autosampler. After the injection of 50 μL, analytes were separated by use of a column saver (2.0 μm filter, Supelco Analytical, Seelze, Germany), a security guard cartridge (C_{18}, 4 mm × 2 mm, Phenomenex, Aschaffenburg, Germany), and a Zorbax Eclipse XDB-C_{18} column (50 mm × 4.6 mm, 1.8 μm, Agilent Technologies, Böblingen, Germany) at 25 °C. Eluent A consisted of a 3:97 (v/v) methanol/water mixture containing 50 mM ammonium acetate and 0.1% (v/v) acetic acid, and eluent B consisted of a 97:3 (v/v) methanol/water mixture containing 50 mM ammonium acetate and 0.1% (v/v) acetic acid. The flow rate was 0.4 mL/min throughout the chromatographic run. A linear gradient from 100% (v/v) A to 50% (v/v) B was applied between 0 and 5 min followed by re-equilibration of the column at 100% (v/v) A from 5 to 7 min. Tenofovir was used as an internal standard at a final concentration of 50 ng/mL.

Mass detection was performed on an API 3000 triple quadrupole mass spectrometer (ABSciex, Darmstadt, Germany) using selected ion monitoring (SRM) analysis in positive ionization mode. The SRM transitions were detected with a 40 ms dwell time. Parameters of HPLC−MS/MS fragments are listed in Table 1. Ion source settings and collision gas pressure were manually optimized regarding ion source voltage, ion source temperature, nebulizer gas, and curtain gas (ion source voltage of 5500 V, ion source temperature of 350 °C, curtain gas of 15 psi, and collisionally activated dissociation gas of 10 psi). Chromatographic data were collected and analyzed with Analyst version 1.4.1 (ABSciex). Quantitation was performed with nitrogen as the collision gas.

Radioactive GC Assay. The sGC activities by means of [α-^{32}P]GTP were assayed as described for a nonradioactive NC assay except that 0.3 μCi of [α-^{32}P]GTP was added per assay. Samples were supplemented with 50 μL of 2.2 M hydrochloric acid and centrifuged for 10 min at 20000*g* to sediment denatured protein. To separate [^{32}P]cGMP from the remaining [α-^{32}P]GTP, we loaded samples onto columns containing 1.4 g of neutral aluminum oxide and eluted [^{32}P]cGMP using 100 mM Tris-HCl buffer (pH 7.5).[23] Čerenkov radiation was measured with a Tri-Carb 2810 TR liquid scintillation analyzer (Perkin-Elmer) for 1 min.

Statistics. Data are presented as means ± the standard error of the mean (SEM) and are based on at least six independent experiments. GraphPad Prism version 5.01 (GraphPad, San Diego, CA) was used for nonlinear regression and calculation of mean, SEM, K_i, K_M, V_{max}, and IC_{50} values.

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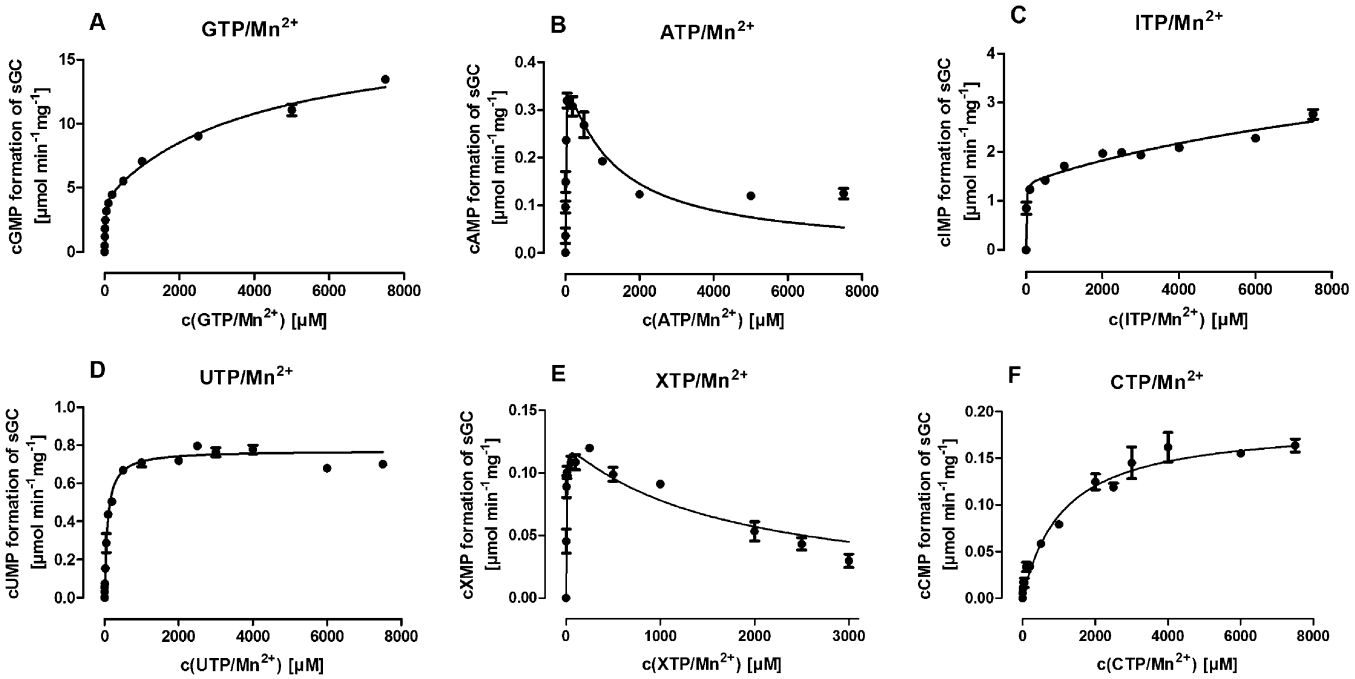

Figure 1. Kinetics of formation of cNMP by sGC in the presence of Mn^{2+}. sGC (0.1−20 ng of sGC per tube) was incubated with 100 μM SNP and various concentrations of NTP/Mn^{2+} at 37 °C. After 5−20 min, depending on the investigated NTP, reactions were stopped by heat and analyzed via HPLC−MS/MS. Data were plotted by use of nonlinear regression. For GTP (A) and ITP (C), a model based on two substrate binding sites was used. For ATP (B) and XTP (E), data were best described using a substrate inhibition model. Results for UTP (D) and CTP (F) were best described by monophasic modeling. Please note in all panels the different scales of the *y*-axes and in (E) the different *x*- and *y*-axes. Data shown are the means ± SEM of six independent experiments. The results of the nonlinear regression analysis of data are listed in Table 2.

Table 2. Michaelis−Menten Kinetic Parameters of sGC for Various NTPs Using Mn^{2+} as a Cofactor[a]

	ATP	CTP	GTP	ITP	TTP	UTP	XTP
K_{M1} (μM)	14.8 ± 0.2	1144 ± 148	14.2 ± 1.6	6.6 ± 1.9	nd	80.8 ± 6.2	2.5 ± 0.5
V_{max1} (μmol min^{-1} mg^{-1})	0.40 ± 0.02	0.19 ± 0.01	4.1 ± 0.15	1.4 ± 0.1	nd	0.77 ± 0.01	0.1 ± 0.01
K_{M2} (μM)	–	–	4819 ± 552	13189	nd	–	–
V_{max2} (μmol min^{-1} mg^{-1})	–	–	13.5 ± 0.7	3.4 ± 1.5	nd	–	–
V_{max1} of GC (%)	9.8	4.6	100	34.1	–	18.8	2.4
K_i (μM)	1167 ± 213						1696 ± 276

[a]NC activities were analyzed as described in Materials and Methods. sGC (1−20 ng per tube) was incubated with 2−7500 μM NTP/Mn^{2+} (2−3000 μM XTP) in the presence of 100 μM SNP and 3 mM Mn^{2+} ions at 37 °C for 5−20 min depending on the analyzed NTP. Apparent K_M, K_i, and V_{max} values represent the means ± SEM of at least six independent experiments shown in Figure 1 and are given in alphabetical order of NTPs. Curves were analyzed by nonlinear regression using Prism version 5.0. nd, not detected.

RESULTS

Comparison of the Radioactive Cyclase Assay versus the HPLC−MS/MS-Based Cyclase Assay. NC assays are routinely performed using methods based on radiolabeled substrates or antibodies.[9,15,24,25] We developed an HPLC−MS/MS method for the simultaneous quantitation of seven cNMPs. To compare the radioactive and nonradioactive quantitation method, we incubated 1 ng of sGC per tube for 20 min at 37 °C with various concentrations of GTP/Mn^{2+}. Figure S1 of the Supporting Information illustrates that both methods achieved nearly identical Michaelis−Menten plots. The GC activity of sGC showed a saturation plot with a K_M of 28.8 ± 3.7 μM and a V_{max} of 3.7 ± 0.1 μmol min^{-1} mg^{-1} for the radioactive assay and a K_M of 47.1 ± 4.7 μM and a V_{max} of 3.6 ± 0.1 μmol min^{-1} mg^{-1} for the HPLC−MS/MS method. Having validated the HPLC−MS/MS method, we performed all further experiments via this approach.

Time Courses of sGC Activity. Incubation of 0.1 ng of sGC per tube and 200 μM GTP/Me^{2+} in the presence of 3 mM free Me^{2+} ions and 100 μM SNP at 37 °C resulted in linear cGMP production for 90 min (Figures S2A and S3A of the Supporting Information). As expected, sGC showed a large increase in the level of GTP cyclization. The activity of sGC was 20-fold higher in the presence of Mn^{2+} than in the presence of Mg^{2+}. However, other substrates were also accepted, although activities were significantly lower. In the presence of Mg^{2+}, sGC converted GTP, ATP, ITP, and XTP. In the presence of Mn^{2+}, sGC additionally cyclized UTP and CTP. In comparison to GTP, other NTPs showed a distinct time-dependent saturation. Specifically for NTPs other than GTP, the linearity of reaction was lost after 5−20 min, depending on the NTP studied. For both experimental conditions, a thymidinyl cyclase (TC) activity was not observed. All further experiments were performed under linear conditions to yield accurate Michaelis−Menten plots.

Enzyme Kinetics of sGC. We analyzed the Michaelis−Menten kinetics of sGC for GTP, ATP, ITP, UTP, CTP, and XTP in a concentration range of 2−1000 μM in the presence of

Table 3. Michaelis–Menten Kinetic Parameters of sGC for Various NTPs Using Mg^{2+} as a Cofactor[a]

	ATP	CTP	GTP	ITP	TTP	UTP	XTP
K_M (μM)	5548 ± 1.12	nd	119.1 ± 13.7	796 ± 90	nd	nd	241 ± 27
V_{max} (μmol min^{-1} mg^{-1})	0.9 ± 0.1	nd	2.2 ± 0.5	4.9 ± 0.1	nd	nd	0.008 ± 0.0003
V_{max} of GC (%)	40.9	–	100	222	–	–	0.4

[a]NC activities were analyzed as described in Materials and Methods. sGC (1–50 ng per tube) was incubated with 2–7500 μM NTP/Mg^{2+} (2–3000 μM XTP) in the presence of 100 μM SNP and 3 mM Mg^{2+} ions at 37 °C for 5–10 min depending on the analyzed NTP. Apparent K_M, K_i, and V_{max} values represent the means ± SEM of at least six independent experiments shown in Figure 2 and are given in alphabetical order of NTPs. Curves were analyzed by nonlinear regression using Prism version 5.0. nd, not detected.

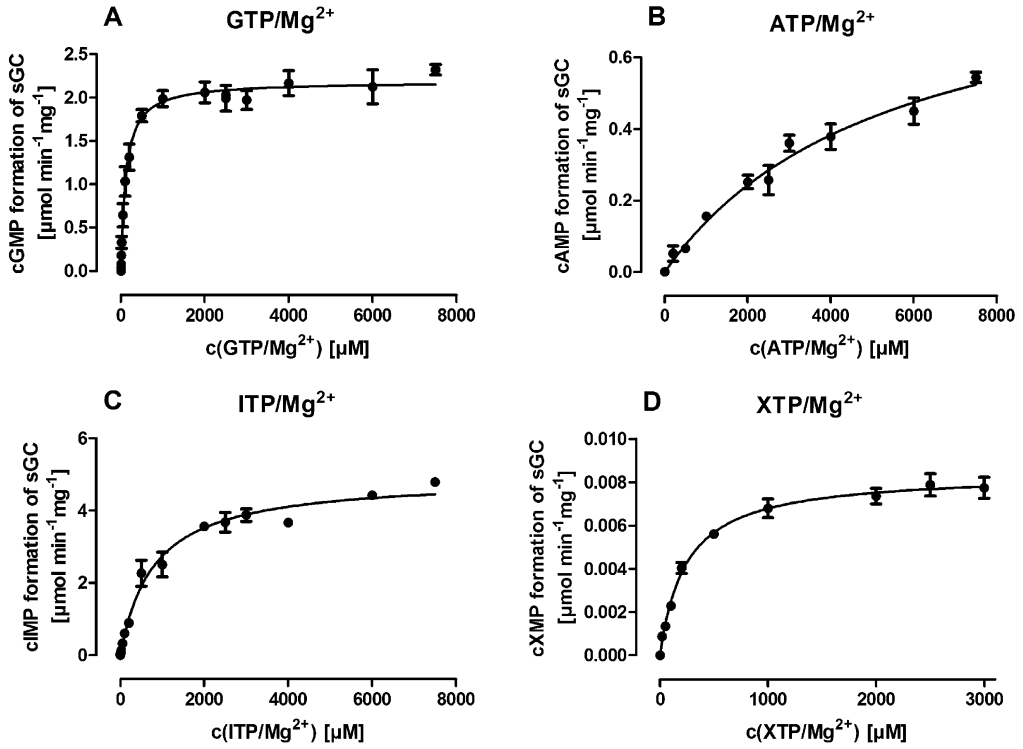

Figure 2. Kinetics of formation of cNMP by sGC in the presence of Mg^{2+}. sGC (1–50 ng per tube) was incubated with 100 μM SNP and various concentrations of NTP/Mg^{2+} at 37 °C. After 5–20 min, depending on the investigated NTP, reactions were stopped with heat and analyzed via HPLC–MS/MS. Data were plotted by use of nonlinear regression. For all panels, data were best described using a monophasic model. A pyrimidinyl cyclase activity was not detected. Please note in all panels the different scales of the *y*-axes and in panel D the different *x*- and *y*-axes. Data shown are the means ± SEM of six independent experiments. The results of the nonlinear regression analysis of data are listed in Table 3.

3 mM free Mn^{2+} and 100 μM SNP. With regard to V_{max}, we observed a descending order of cNMP formation: cGMP > cIMP > cUMP > cAMP > cCMP ≈ cXMP (Figure 1). With regard to K_M, we found an ascending order: XTP < ITP < GTP ≈ ATP ≪ UTP ≪ CTP. ATP and XTP exhibited substrate inhibition, and XTP and ITP showed a considerably lower K_M than GTP. A summary of the kinetic properties of sGC is shown in Table 2. In the presence of Mn^{2+} and in the case of GTP and ITP, we additionally observed that catalytic activity in the high micromolar concentration range (500–1000 μM) was not appropriately described by using a single-site binding model. Therefore, we additionally investigated saturation behavior of all NTPs in the millimolar concentration range (1000–7500 μM; for XTP, 1000–3000 μM). For GTP and ITP, data points were better described by use of a model with a biphasic saturation curve (Figure 1 and Table 2). For pyrimidine NTPs, curve fitting using a monophasic regression curve was sufficient even at millimolar concentrations.

When Mn^{2+} was replaced with Mg^{2+}, the NC activity of sGC was reduced except for that of ITP, and K_M was increased for all NTPs. With regard to V_{max}, we observed a descending order of cNMP formation: cIMP > cGMP ≫ cAMP ≫ cXMP (Table 3). For K_M values, we found an ascending order: GTP < XTP < ITP ≪ ATP. In the case of GTP, GC activity decreased to half of the activity with Mn^{2+}, and K_M increased 8-fold. Surprisingly, for ITP in the presence of Mg^{2+}, V_{max} was even 2-fold higher than for GTP. For all NTPs, best fits were achieved by Michaelis–Menten plots including only one binding site. A pyrimidinyl cyclase activity in the presence of Mg^{2+} was not observed.

Inhibition of sGC's GC Activity by ATP. The substrate inhibition curves for ATP indicated an inhibitory effect on cAMP synthesis (Figure 1B). ATP is a mixed-type inhibitor for NO-stimulated sGC activity.[15] We analyzed the influence of ATP on 1 mM GTP cyclization by sGC (0.1–1 ng per tube) at 37 °C for 20 min in the presence of Mn^{2+} or Mg^{2+}. As illustrated in Figure S4A of the Supporting Information, ATP reduced GC activity with an IC_{50} of 204.7 ± 1.2 μM in the presence of Mn^{2+} and an IC_{50} of 252.4 ± 1.0 μM in the presence of Mg^{2+}. Moreover, even in the presence of 1 mM GTP, an AC activity was observed (Figure S4B of the Supporting Information). Kinetic studies revealed a substrate

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Table 4. Substrate Specificity of sGC for Various NTP/Me^{2+} Forms under Basal, NO-Activated, and Heme-Oxidized Conditions[a]

		ATP	CTP	GTP	ITP	TTP	UTP	XTP
Mn^{2+}	basal	1.7 ± 0.2	1.1 ± 0.1	661.7 ± 58.9	36.6 ± 1.5	nd	2.5 ± 0.8	0.7 ± 0.08
	SNP	240.8 ± 20.0	8.9 ± 2.1	3272 ± 464	1232 ± 35	nd	92.4 ± 8.6	16.2 ± 6.5
	ODQ and SNP	1.6 ± 0.1	0.3 ± 0.04	965 ± 120	39.3 ± 1.4	nd	2.4 ± 0.2	0.6 ± 0.1
Mg^{2+}	basal	nd	nd	28 ± 0.01	5.2 ± 0.01	nd	nd	nd
	SNP	4.4 ± 0.4	nd	2850 ± 108	1323 ± 38	nd	nd	0.4 ± 0.1
	ODQ and SNP	nd	nd	20 ± 0.6	5.6 ± 0.6	nd	nd	nd

[a]NC activities were analyzed as described in Materials and Methods. sGC (50 ng per tube) was incubated with 200 μM NTP/Me^{2+} in the presence of 100 μM SNP or 100 μM ODQ with SNP and 3 mM Me^{2+} ions at 37 °C for 60 min. Values represent the means ± SD of at least two independent experiments performed in duplicate and are given in alphabetical order of NTPs and in units of nanomoles per minute per milligram. nd, not detected. Please note that experiments were not performed under linear conditions.

inhibition with the following parameters for Mn^{2+}: K_M = 306 ± 88.8 μM, V_{max} = 0.23 ± 0.04 μmol min^{-1} mg^{-1}, and K_i = 1094 ± 329 μM. For Mg^{2+}, the determination of kinetic parameters was ambiguous. Finally, our data are in accord with those published for inhibition of sGC via ATP.[15]

Substrate Specificity of Basal and Ferric sGC. To investigate whether sGC's nucleotidyl cyclase activity can also be observed under basal conditions and with ferric sGC, we incubated 50 ng of sGC per tube and 200 μM NTP/Me^{2+} in the presence of 3 mM free Me^{2+} ions at 37 °C for 60 min. As documented in Table 4, sGC exhibited basal nucleotidyl cyclase activity for GTP, ATP, ITP, CTP, UTP, and XTP with Mn^{2+} as the divalent cation. No TC activity was detected. The highest basal activity in the presence of Mn^{2+} was found for GTP, followed by ITP, UTP, ATP, and XTP. In the presence of Mg^{2+}, basal activities were substantially lower and NC activities could be detected for only GTP and ITP. SNP exhibited differential effects on basal activities. In the presence of Mn^{2+}, SNP increased GC activity 5-fold. The strongest stimulation was found for AC activity (142-fold) followed by UC activity (37-fold), IC activity (33-fold), XC activity (23-fold), and CC activity (8-fold). In the presence of Mg^{2+}, stimulation of GC activity by SNP was 100-fold and that for IC activity 254-fold. For CTP and UTP in the presence of Mg^{2+}, no pyrimidinyl cyclase activity was observed. However, with ATP and XTP, some SNP-stimulated activity was observed in the presence of Mg^{2+}. Treatment of sGC with 100 μM ODQ oxidizes Fe^{II} of the heme moiety to Fe^{III}, resulting in NO insensitivity.[8] ODQ reduced SNP-stimulated NC activities and did so differentially. In the presence of Mn^{2+}, ODQ reduced CC activity to one-third of basal activity, whereas in the case of GTP and ITP, a small stimulatory effect of SNP was still observed. With ATP, UTP, and XTP, NC activity was reduced slightly below basal values. In the presence of Mg^{2+}, ODQ abrogated SNP-stimulated AC and XC activity. Under these conditions, GC activity was reduced below the basal level, and in the case of IC activity, a small stimulatory effect of SNP was still evident.

DISCUSSION

Comparison of the Radioactive Cyclase Assay versus the HPLC–MS/MS-Based Cyclase Assay. The NC activity of GC has always been highly controversial on the basis of impure enzyme preparations, analytical standards, or the cross reactivity of the used antibodies.[12,16−21] Even today, quantitation of cAMP and cGMP followed by an enzyme immunoassay can be problematic, particularly with regard to cross reactivity.[22] A further problem with radiometric assays is the isotope dilution with higher NTP concentrations followed by a loss of sensitivity.[15] This loss of sensitivity can be partially compensated by drastically increasing the amount of radioactively labeled NTP, but such a methodological procedure does not find broad acceptance among scientists. Therefore, we established a quantitation method based on HPLC–MS/MS with high sensitivity and selectivity. In contrast to the methods mentioned above, for this technique, all NTPs are commercially available and do not have to be radiolabeled. Our method also allows for unequivocal molecular identification of any given cNMP by specific ratios of molecule quantifiers and identifiers. Importantly, higher substrate concentration ranges can easily be analyzed because an isotope dilution is missing. As illustrated in Figure S1 of the Supporting Information, our HPLC–MS/MS method achieved results very similar to those of the classical, radioactive assay.

Time Courses of sGC Activity. Taking advantage of the HPLC–MS/MS system, we investigated the substrate specificity of sGC. To receive exact results, we used only enzyme preparations of the highest available purity.[23] By investigating the time-dependent generation of cNMPs, we could confirm that GTP cyclization is linear over time for at least 90 min[19] and cGMP synthesis is the most effective reaction compared to other cyclization reactions. However, sGC accepted further substrates. The formation rate in descending order was as follows: cGMP ≫ cIMP > cAMP > cUMP > cXMP > cCMP. In contrast to cGMP, other cNMPs showed a distinct time-dependent saturation of cNMP formation. This saturation was not due to a lack of substrate as nucleotide concentrations were well above the K_M. Apparently, GTP is the most effective NTP in terms of stabilization of a catalytically active sGC: GTP > ITP > ATP > UTP > CTP > XTP. In the case of Mg^{2+}, sGC's substrate specificity was reduced to purine NTPs (GTP, ATP, ITP, and XTP). The stabilization effectiveness of NTPs in the presence of Mg^{2+} in descending order is as follows: GTP ≫ ITP > ATP ≈ XTP. In general, with purine NTPs, we observed a higher cyclase activity than with pyrimidine NTPs in terms of V_{max}, which is probably due to the strong structural similarity to GTP. Under all experimental conditions, a TC activity was not observed. In comparison to other NTPs, dTTP lacks the 2′-hydroxyl group at the ribosyl moiety. However, Garbers et al.[16] showed that the supernatant fraction of rat lung preparations formed 2′-deoxyguanosine 3′,5′-cyclic monophosphate from dGTP with 50% effectiveness compared to GC activity. The combination of a missing 2′-hydroxyl group and a missing purine base is probably responsible for a lack of activity of sGC on dTTP.

Enzyme Kinetics of sGC. So far, no X-ray crystal structure of full-length sGC has been determined. Current sGC models

Table 5. Comparison of Reported Michaelis−Menten Kinetic Parameters of sGC for NTPs

substrate	K_M (μM)	V_{max} (μmol min^{-1} mg^{-1})	activator	source	method	ref
GTP/Mn^{2+}	15	0.29	SNP	bovine lung	radiometric	9
GTP/Mn^{2+}	–	0.9−1.8	SNP	bovine lung	radiometric	35
GTP/Mn^{2+}	10	0.1	NO gas	bovine lung	radiometric	37
GTP/Mg^{2+}	44	11.8	DEA/NO	$rat_{rec.}$ in Sf9 cells	immunoassay	15
GTP/Mg^{2+}	156	0.75	SNP	bovine lung	immunoassay	25
GTP/Mg^{2+}	21	1.396	SNAP	$rat_{rec.}$ in Sf21 cells	radiometric	29
GTP/Mg^{2+}	–	1.2−2.4	SNP	bovine lung	radiometric	35
GTP/Mg^{2+}	85−120	0.4	NO gas	bovine lung	radiometric	37
GTP/Mg^{2+}	49.8	11.642	NOC-12	$human_{rec.}$ in Sf9 cells	HPLC−UV	30
GTP/Mg^{2+}	119	1.49	DEA/NO	$rat_{rec.}$ in Sf9 cells	radiometric	31
GTP/Mg^{2+}	43	1.330	NOC-12	bovine lung	HPLC−UV	32
GTP/Mg^{2+}	22	11.5	NO gas	bovine lung	radiometric	33
GTP/Mg^{2+}	–	28.2	NO gas	bovine lung	immunoassay	34
ATP/Mn^{2+}	15	0.015	SNP	bovine lung	radiometric	9
ATP/Mg^{2+}	52	0.63	DEA/NO	$rat_{rec.}$ in Sf9 cells	immunoassay	15
ITP/Mn^{2+}		10% of GC[a]	–	rat lung	radiometric	16
UTP/Mn^{2+}	25	0.0092	SNP	bovine lung	radiometric	9

[a]Substrate concentration of 0.1 mM. Abbreviations: DEA/NO, diethylamine nonoate; NOC-12, *N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)-ethanamine; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; rec., recombinant.

are based on crystal structures of the catalytic domain.[26,27] The catalytic site is localized in the C-terminus of the α- and β-subunits. Both domains are required for catalytic activity. sGC from *Chlamydomonas reinhardtii* contains two active sites that act cooperatively, providing evidence that the active sites interact with each other.[27] However, in the second binding site, basic residues, which are responsible for coordinating β- and γ-phosphate binding of NTPs and for maintaining the transition state, and two aspartate residues, which are required for metal coordination, are missing. Thus, this binding site is predicted to lack catalytic activity.[27,28] Therefore, sGC is suggested to exhibit a nucleotide-like (pseudosymmetic) binding site and a putative catalytic site.[15]

By using highly purified sGC and HPLC−MS/MS quantitation, we could show that in vitro, sGC has broader substrate specificity than previously assumed. As expected, we confirm that the dominant cyclase activity of sGC was the GC activity. We found kinetic parameters (K_M = 14.2 μM, and V_{max} = 4.1 μmol min^{-1} mg^{-1}) in the presence of Mn^{2+} comparable to literature kinetic parameters with a K_M within the range of 10−156 μM and a V_{max} with the range of 0.75−28.2 μmol min^{-1} mg^{-1}[15,25,29−37] (Table 4). We also could confirm that using Mg^{2+} ions, the specific activity of rat sGC is ∼50% lower than that of bovine sGC.[23]

In the presence of Mn^{2+}, ATP, CTP, ITP, UTP, and XTP were substrates of sGC, too. In comparison to that of GTP, V_{max} values of the other NTPs were significantly lower. An AC activity of bovine lung sGC has been described by Gille et al. with a K_M of 15 μM and a V_{max} of 15 nmol min^{-1} mg^{-1}.[9] We found a comparable K_M of 14.8 μM but a significantly higher V_{max} of 400 nmol min^{-1} mg^{-1}. This difference may be explained by different incubation temperatures (30 °C vs 37 °C), different enzyme sources, or longer periods of storage of bovine sGC. Gille et al. also reported a significantly lower V_{max} for GC activity (Table 5), which he suggested was due to the specific commercial enzyme preparation used.[9] Gerzer et al. showed that at 37 °C the specific AC activity of bovine sGC was 630 nmol min^{-1} mg^{-1} in the presence of Mn^{2+}.[19] Moreover, we could confirm that ATP exhibits substrate inhibition at millimolar concentrations.[15]

Gille et al. also described a bona fide UC activity with a K_M of 25 μM and a V_{max} of 9.2 nmol min^{-1} mg^{-1} in a radiometric assay.[9] We determined a K_M of 80.8 μM and a significantly higher V_{max} of 770 nmol min^{-1} mg^{-1} using the structure-based HPLC−MS/MS assay. Besides GC, AC, and UC activity, we found that sGC also catalyzed the formation of cIMP with a K_M of 6.6 μM and a V_{max} of 1.4 μmol min^{-1} mg^{-1}. An IC activity of sGC was formerly described by Garbers et al. amounting to 10% of the GC activity using 0.1 mM ITP, but kinetic parameters were not determined.[16] These parameters are first described here. The differences can be explained by the facts that Garbers et al. did not stimulate sGC by any NO donor and reactions were conducted at 30 °C. The high IC activity documented in this paper explains that Chang et al. reported a considerable inhibition of GC activity by ITP.[29] Interestingly, the V_{max} of IC activity was not largely affected when Mn^{2+} was replaced with Mg^{2+} and cyclase activity was even higher than for GTP. We also found for the first time that in the presence of Mg^{2+} and Mn^{2+} sGC accepted XTP as a substrate and in the presence of Mn^{2+} sGC accepted CTP. Although the CC and XC activity of sGC has not yet been reported, probably because it is low, Zwiller et al.[13] demonstrated that CTP and UTP inhibited GC activity in a competitive manner. This can be explained by the fact that CTP and UTP bind to the catalytic site and are substrates of sGC.

We found that for GTP and ITP saturation data in the presence of Mn^{2+} ions were best fitted using a model with two binding sites. When Mn^{2+} ions were replaced with Mg^{2+} ions, catalysis became monophasic. Indeed, it is known that one sGC molecule binds two molecules of GTP in the presence of Mn^{2+} using equilibrium dialysis.[38] Yazawa and co-workers[38] suggested a high-affinity site serving as catalytic site and a low-affinity site for regulation. Derbyshire et al.[15] showed that the NO-independent activator of sGC YC-1 and ATP bind to distinct sites and that the allosteric nucleotide binding site is located at the C-terminus of sGC. We could confirm that sGC possesses two nucleotide binding sites. We suggest that the catalytic site accepts ITP, ATP, XTP, UTP, and CTP apart from GTP. A second allosteric binding site regulates sGC activity. Binding of ATP and XTP in the millimolar concentration range to the

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allosteric site results in an inhibition of the catalytic domain. Binding of GTP and ITP to the allosteric site leads to a stabilization of the active confirmation, so that sGC exhibits higher GC and IC activity. Figure 3 illustrates formation of cNMP by sGC according to the two-site model.

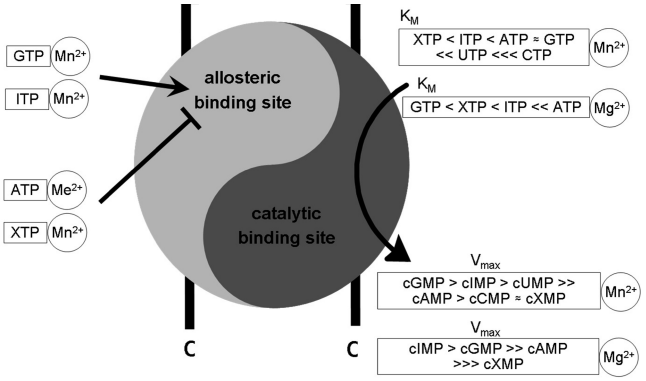

Figure 3. Model of the sGC substrate affinity and catalytic activity. Illustrated is the C-terminus of sGC that contains two nucleotide binding sites. Depending on the divalent cation, sGC exhibits distinct K_M values for NTPs at the catalytic domain in ascending order as indicated. Additionally, sGC has an allosteric (pseudosymmetric) binding site that is regulated by ATP/Me^{2+}, XTP/Mn^{2+}, ITP/Mn^{2+}, and GTP/Mn^{2+} in the millimolar concentration range. ATP/Me^{2+} and XTP/Mn^{2+} inhibit the catalytic domain, while GTP/Mn^{2+} and ITP/Mn^{2+} stabilize the GC and IC activity of sGC, respectively.

Substrate Specificity of Basal and Ferric sGC. NC activity could also be unequivocally detected under basal conditions and with ferric sGC (Table 4), although large amounts of enzyme were required. Under basal and activated conditions, we observed the same order of cNMP formation as shown in Figures S2 and S3 of the Supporting Information. However, depending on the analyzed NTP, sGC exhibited distinct profiles of activation by SNP. The substrate-dependent stimulation of sGC by NO is a first hint of the notion that in intact cells, sGC discriminates among various substrates and that the resulting cNMPs possess different functions. This concept is further corroborated by the finding that the oxidant ODQ differentially affected NC activities. The differential effects of ODQ depended also on the specific cation used. Apparently, heme oxidation in sGC results in a propagated conformational change to the catalytic site that differentially interferes with the binding and turnover of specific NTPs. Thus, ATP, ITP, XTP, CTP, and UTP are not just simple substitutes for GTP at sGC but substrates with specific functional properties in their own right. Future studies will have to elaborate the precise kinetic parameters of basal and ODQ-inhibited NC activities of sGC with various NTPs. We anticipate that this analysis will unmask very intriguing differences between the various NTPs. However, these studies will be quite challenging because high sGC concentrations will be needed for several substrates, and linear conditions will have to be ensured. The sensitivity of the mass spectrometer used in this study (API 3000, ABSciex) will probably not be sufficient for all analyses, and we may have to switch to the more sensitive 5500 QTrap instrument (ABSciex). According to our experience with cNMP quantitation, the 5500 QTrap instrument is 5−10-fold more sensitive than the API 3000 instrument.

Context of the CC Activity of sGC with Previously Reported CC Activities. The existence of a mammalian CC activity was suggested 35 years ago,[39] but there were substantial problems with the separation of educt and product on the alumina columns.[40] These problems, together with problems related to the questionable specificity of cCMP-detecting antibodies (reviewed in ref 41), have resulted in very little research of CC. The biochemical properties of previously reported tentative CC activities[42−44] do not fit with the properties of sGC reported here. It should be noted that the previously performed studies of CC activity were performed with crude enzyme preparations. Thus, there could have been multiple forms of interference with the detection methods used, and the molecular identity of the CC had remained elusive in the previous studies. To the best of our knowledge, this is the first report describing a CC activity of a highly purified, well-validated, and clearly defined mammalian enzyme tested previously in other studies.[23,45]

Using highly purified bacterial "adenylyl" cyclase toxins as model NCs, we have recently developed a sensitive and specific radiometric method for the detection of cCMP, cUMP, and cIMP formation.[24] When very high concentrations of toxins are used, cCMP, cUMP, and cIMP formation can even be detected by HPLC.[24] As a further advancement, here, we developed a sensitive and specific combined HPLC−MS/MS method for the detection of cCMP, cUMP, and cIMP formation. With this methodology, we unequivocally showed that highly purified soluble "guanylyl" cyclase exhibits broader substrate specificity than previously assumed. Most strikingly, sGC synthesizes cCMP and cUMP in the presence of Mn^{2+}. Notably, too, sGC generates relatively large amounts of cIMP in the presence of Mn^{2+} and Mg^{2+}. Thus, our data raise the question of whether the rather broad purinyl and pyrimidinyl cyclase activity of sGC is relevant in intact cells.

Possible Roles of cCMP, cUMP, and cIMP as Second Messengers. A previous study suggested the physiological existence of cCMP, cUMP, and cIMP in tissues using fast atom bombardment mass spectrometry,[46,47] but the precision and specificity of this method may have been insufficient for unequivocal detection of these cyclic nucleotides. So far, the results of the Newton group have not yet been confirmed independently by another group. In addition, cNMP quantitation is not possible with fast atom bombardment. It will be important to assess cellular cCMP and cUMP concentrations in direct comparison with cAMP and cGMP concentrations. Immunological methods are problematic.[41] However, the HPLC−MS/MS method described here is suitable for quantitation and identification of cNMPs, using specific qualifiers and quantifiers for any given cNMP (Table 1). In forthcoming studies, we will apply our HPLC−MS/MS method to the detection of cCMP, cUMP, and cIMP in intact cells. Because of the clear discrimination among all cNMPs in terms of HPLC retention time and MS/MS fragmentation patterns, we now have the option to simultaneously detect and quantitate seven cNMPs (cAMP, cCMP, cGMP, cIMP, cTMP, cUMP, and cXMP).

cCMP was thought to be involved in the regulation of cell growth, proliferation, tissue development, and modulation of immune responses, but several issues such as questionable membrane penetration of cCMP and very low cCMP concentrations (as low as 100 nM) used to elicit biological effects hampered the interpretation of data and resulted in overall skepticism in the scientific community (reviewed in ref

41). Later, a membrane-permeable cCMP analogue at rather high concentrations (300 μM to 1 mM) was shown to regulate the production of superoxide anion in human neutrophils in a stimulus-dependent manner, but the underlying mechanisms remained elusive.[48] Additionally, the purity of the previously used cCMP analogue was of concern. More recently, Desch et al.[49] have shown that a highly purified membrane-permeable cCMP analogue induces vascular smooth muscle relaxation via cGMP-dependent protein kinase and the IRAG protein. Evidence of this mechanism was convincingly provided by the use of corresponding gene knockout animals. Additionally, cCMP and cUMP show different manners of degradation by various purified mammalian phosphodiesterases.[50] Most strikingly and in marked contrast to cUMP, cCMP is resistant to degradation by all phosphodiesterases examined so far. This finding could imply that even with low rates of generation of cCMP by a cCMP-forming enzyme (such as sGC), substantial cellular cCMP concentrations could build up, allowing for substantial biological effects to occur. Moreover, cCMP and cUMP differentially activate cGMP-dependent protein kinase and two isoforms of cAMP-dependent protein kinase.[51] Intriguingly, several publications described an activation of cAMP signaling pathways following NO activation of sGC in intact cells[52−54] and elimination of cGMP shows complex interaction with cAMP.[55] A responsible enzyme has not yet been found, but with respect to our data, sGC's AC activity is a likely candidate. In support of the possible physiological relevance of pyrimidinyl cyclase activity of sGC, the bacterial adenylyl cyclase toxins CyaA from *Bordetella pertussis* and edema factor from *Bacillus anthracis* also possess CC and UC activity.[24] Moreover, dissociations between edema factor-induced cAMP accumulation and biological effects have been observed in several systems, lending support to the concept that second messengers other than cAMP contribute to the toxin effects.[56]

Role of Mn^{2+} in sGC Regulation. At first glance, Mn^{2+} is unlikely the substrate cation cofactor of sGC because the concentration of intracellular Mg^{2+} in vivo is some 2−3 orders of magnitude higher than that of Mn^{2+}.[36] However, Mn^{2+} also plays a critical role for important enzymes such as pyruvate carboxylase[57] and mitochondrial superoxide dismutase.[58] While Mn^{2+} toxicity in humans is well-documented, very little is known about the symptoms of Mn^{2+} deficiency, which is also due to methodological problems.[59] However, studies with hemodialysis patients suggest that Mn^{2+} deficiency is associated with increased mortality.[60] Additionally, a sufficient Mn^{2+} supply is important for proper fetal development.[61]

Mn^{2+} may be present at higher concentrations in vivo in distinct compartments or microdomains in which sGC resides. The α_2 subunit of sGC has already been shown to be enriched in the postsynaptic site of neurons.[62] For soluble AC, it is known that cAMP signaling is regulated in discrete intracellular compartments such as focal cytoplasm points, mitochondria, and the nucleus.[63] Additionally, phosphodiesterases have been shown to limit the spread of cAMP and cGMP in shaping and organizing intracellular signaling microdomains.[64] The striking Mn^{2+} dependency of formation of cCMP and cUMP by purified sGC provides us with a unique opportunity to assess the possible physiological relevance of Mn^{2+} in sGC regulation in intact cells. Specifically, if Mn^{2+} is physiologically relevant for sGC in intact cells, we then expect NO-stimulated formation of cCMP and cUMP to occur. If Mg^{2+} is physiologically relevant for sGC in intact cells, we then expect no NO-stimulated formation of cCMP and cUMP to occur. With our newly developed HPLC−MS/MS method, we will address these highly important questions is a forthcoming study. These studies will be fundamentally important for the field of NCs in general because so far, for no enzyme of this class has the question of the physiological relevance of Mn^{2+} or Mg^{2+} as a cofactor been resolved. However, striking differential impacts of Mg^{2+} and Mn^{2+} have also been observed for the activation and inhibition of mammalian membranous ACs by diterpenes.[65] Also, from the perspective of Mn^{2+} as an important trace element in the human body,[60,61] the differential regulation of formation of cNMP by sGC provides a unique opportunity to address this as yet poorly understood issue.

Some Future Studies. Further studies will have to elucidate if sGC isoform $\alpha_2\beta_1$, which is predominately expressed in brain and fetal tissue, also exhibits NC activity. sGC isoforms $\alpha_1\beta_1$ and $\alpha_2\beta_1$ contain equivalent amounts of heme, exhibit comparable kinetic properties, and exhibit very similar sensitivity toward NO.[5] As has been observed for the $\alpha_1\beta_1$ isoform, ODQ inhibited and YC-1 stimulated and sensitized the $\alpha_2\beta_1$ enzyme toward NO and carbon monoxide.[5] However, binding of NTPs other than GTP and NC kinetic parameters have not yet been assessed for $\alpha_2\beta_1$. Future studies also have to investigate the substrate specificity of the membrane-bound GCs (pGCs). Although they are highly homologous in the catalytic domains to sGC,[4] there are some differences to note. pGCs are homodimers and are regulated by peptide and ATP binding. In contrast to sGC, both subunits constitute catalytically active domains that act via positive cooperativity.[66] Particulate fractions from sea urchin sperm and crude membranes obtained from homogenates of rat kidney have already been demonstrated to generate cIMP[16] and cAMP,[20] respectively. However, analysis of a highly purified membrane preparation concerning NC activity is still missing. On the basis of our data for sGC, we predict that pGCs also exhibit substantial NC activities.

Crystallization of holo-sGC is an ambitious goal, and so far, the efforts of several leading laboratories in this field have been unsuccessful. Our results should aid the crystallization of sGC. Previous crystallization experiments may have failed because of a lack of stabilizing ligands. Specifically, the inclusion of MANT- and/or TNP-substituted cytosine, uracil, and/or hypoxanthine nucleotides may facilitate future crystallization efforts. In the case of mACs, MANT- and TNP-nucleotides turned out to be most valuable ligands for crystallography.[67−69] Moreover, MANT- and TNP-nucleotides are the most valuable tools for studying different active mAC conformations.[11] By analogy, the use of fluorescent nucleotides may also facilitate the analysis of the assumed activation-dependent interaction of sGC with various purine and pyrimidine nucleotides.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Comparison of radiometric and HPLC−MS/MS methods as well as time courses of sGC's NC activity in the presence of Mn^{2+} and Mg^{2+} and its inhibition by ATP in the presence of Mn^{2+} and Mg^{2+}. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ DEDICATION

This publication is dedicated to Prof. Günter Schultz' 75th anniversary. Günter Schultz is a pioneer in cGMP and sGC research.

■ ABBREVIATIONS

AC, adenylyl cyclase; ATP, adenosine 5′-triphosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; CC, cytidylyl cyclase; cCMP, cytidine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cIMP, inosine 3′,5′-cyclic monophosphate; cNMP, nucleoside 3′,5′-cyclic monophosphate; cTMP, thymidine 3′,5′-cyclic monophosphate; CTP, cytosine 5′-triphosphate; cUMP, uridine 3′,5′-cyclic monophosphate; cXMP, xanthosine 3′,5′-cyclic monophosphate; GC, guanylyl cylase; GTP, guanosine 5′-triphosphate; HPLC−MS/MS, high-performance liquid chromatography and tandem mass spectrometry; IC, inosityl cylcase; ITP, inosine 5′-triphosphate; mAC, membranous adenylyl cyclase; MANT-nucleotides, 2′(3′)-*O*-(*N*-methylanthraniloyl) nucleotides; NC, nucleotidyl cyclase; NO, nitric oxide; NTP, nucleoside 5′-triphosphate; ODQ, [1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; SEM, standard error of the mean; SNP, sodium nitroprusside; TC, thymidinyl cyclase; TNP-nucleotides, 2′,3′-*O*-(2,4,6-trinitrophenyl) nucleotides; dTTP, thymidine 5′-triphosphate; UC, uridylyl cyclase; UTP, uridine 5′-triphosphate; XC, xanthosinyl cyclase; XTP, xanthosine 5′-triphosphate.

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