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Differential Inhibition of Various Adenylyl Cyclase Isoforms and Soluble Guanylyl Cyclase by 2′,3′-O-(2,4,6-Trinitrophenyl)-Substituted Nucleoside 5′-Triphosphates

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ABSTRACT

Adenylyl cyclases (ACs) catalyze the conversion of ATP into the second messenger cAMP and play a key role in signal transduction. In a recent study (Mol Pharmacol 70:878–886, 2006), we reported that 2′,3′-O-(2,4,6-trinitrophenyl)-substituted nucleoside 5′-triphosphates (TNP-NTPs) are potent inhibitors (Ki values in the 10 nM range) of the purified catalytic subunits VC1 and IIC2 of membranous AC (mAC). The crystal structure of VC1:IIC2 in complex with TNP-ATP revealed that the nucleotide binds to the catalytic site with the TNP-group projecting into a hydrophobic pocket. The aims of this study were to analyze the interaction of TNP-nucleotides with VC1:IIC2 by fluorescence spectroscopy and to analyze inhibition of mAC isoforms, soluble AC (sAC), soluble guanylyl cyclase (sGC), and G-proteins by TNP-nucleotides. Interaction of VC1:IIC2 with TNP-NDPs and TNP-NTPs resulted in large fluorescence increases that were differentially reduced by a water-soluble forskolin analog, TNP-ATP turned out to be the most potent inhibitor for ACV (Ki, 3.7 nM) and sGC (Ki, 7.3 nM). TNP-UTP was identified as the most potent inhibitor for ACI (Ki, 7.1 nM) and ACII (Ki, 24 nM). TNP-NTPs inhibited sAC and GTP hydrolysis by Gs- and Gi-proteins only with low potencies. Molecular modeling revealed that TNP-GTP and TNP-ATP interact very similarly, but not identically, with VC1:IIC2. Collectively, our data show that TNP-nucleotides are useful fluorescent probes to monitor conformational changes in VC1:IIC2 and that TNP-NTPs are a promising starting point to develop isoform-selective AC and sGC inhibitors. TNP-ATP is the most potent sGC inhibitor known so far.

ACs catalyze the conversion of ATP into the second messenger cAMP and play a key role in signal transduction. In mammals, nine membranous AC isoforms (ACI–IX) have been identified (Defer et al., 2000; Sunahara and Taussig, 2002). mACs differ from each other in regulation and tissue expression, and studies with transgenic and gene-knock-out mice have revealed different functions for the various AC isoforms. Most strikingly, ACV is an important AC isoform in the heart (Göttle et al., 2009), and the knock-out of ACV protects mice against heart failure and induces longevity (Yan et al., 2007). Thus, ACV inhibitors may be valuable drugs for the treatment of cardiovascular diseases and aging (Iwatsubo et al., 2004; Göttle et al., 2009). In addition to mACs, mammals express a structurally distinct sAC that is predominantly found in testis and is important for sperm maturation (Chen et al., 2000). Thus, sAC inhibitors may be promising candidates for male contraceptives (Schlicker et al., 2008). Finally, sGC, catalyzing the conversion of GTP into cGMP, is predominantly expressed in vasculature, and its inhibition is associated with cardiac and respiratory disorders.

ABBREVIATIONS: AC, adenylyl cyclase; mAC, membranous adenylyl cyclase; sAC, soluble adenylyl cyclase; β2AR, β2-adrenoceptor; Gαi, inhibitory G-protein α-subunit; Gαs, short splice variant of the stimulatory G-protein α-subunit; Gαl, long splice variant of the stimulatory G-protein α-subunit; Gαolf, olfactory G-protein α-subunit; sGC, soluble guanylyl cyclase; FPR, formyl peptide receptor; FS, forskolin; GPCR, G-protein-coupled receptor; DMB-FS, 7-acetyl-7-[(N-methylpiperazino) –γ-butyryl]-forskolin; MANT, 2′(3′)-O-(N-methylanthraniloyl); TNP, 2′,3′-O-(2,4,6-trinitrophenyl); VC1 and IIC2, the N- and C-terminal catalytic domains from canine type V mAC and rat type II mAC, respectively, expressed as soluble proteins; GTPγS, guanosine 5′-[(γ-thio)triphosphate].
the second messenger cGMP, is a nucleotidyl cyclase structurally related to mAC (Sunahara et al., 1998). sGC is activated by NO, and NO-containing drugs are effective in the treatment of coronary heart disease and hypertensive crisis (Koesling et al., 2004; Derbyshire and Marietta, 2009; Schmidt et al., 2009). sGC inhibitors may be valuable experimental tools to study the role of this enzyme in cardiovascular and neuronal functions (Gille et al., 2004).

mACs are activated by the G-protein Gαs and require two divalent metal cations for catalysis (Sunahara and Taussig, 2002). ACs I–VIII are also activated by FS (Pinto et al., 2008, 2009). mACs consist of two membrane domains with six transmembrane helices each and two cytosolic domains. The two cytosolic domains, referred to as C1 and C2, form the catalytic core of AC, including the ATP-binding site, the Gαs-binding site, and the Gαi-binding site, whereas the function of the transmembrane domains is as yet poorly defined (Sunahara and Taussig, 2002). Several crystal structures of the cytosolic domains of C1 and C2 (VC1:IIC2) in complex with Gαs, FS, or DMB-FS and various nucleotides have been resolved, providing detailed insight into the mechanisms of catalysis and enzyme inhibition (Tesmer et al., 1997, 2000; Mou et al., 2005, 2006).

MANT-nucleotides (Hiratsuka, 1983; Jameson and Eccleston, 1997) are potent ACV inhibitors and block activation of voltage-dependent calcium channels in cardiomyocytes, but so far, the selectivity of mAC inhibitors is not satisfying (Gille et al., 2004; Rottlaender et al., 2007; Göttle et al., 2009). The MANT-group inserts like a wedge into a hydrophobic pocket between C1 and C2 and prevents transition of AC from the catalytically inactive closed to the catalytically active open conformation (Mou et al., 2005, 2006). Hydrophobic interactions between the MANT-group and C1:C2 pocket contribute very substantially to binding affinity, rendering MANT-nucleotides the most potent mAC inhibitors known so far (Gille et al., 2004; Mou et al., 2005; Göttle et al., 2009). Because the MANT-group is fluorescent and sensitive to changes in hydrophobicity (Hiratsuka, 1983; Jameson and Eccleston, 1997), the interaction of MANT-nucleotides with the hydrophobic pocket provided a unique opportunity to monitor nucleotide binding to and conformational changes of VC1:IIC2 (Mou et al., 2005, 2006; Pinto et al., 2009).

Because MANT-nucleotides are potent AC inhibitors but not particularly mAC isoform-specific, we went on to explore the interaction of other nucleotides with AC. Specifically, in a recent study, we showed that TNP-nucleotides (Hiratsuka, 2003) are also potent mAC inhibitors; i.e., TNP-ATP and TNP-GTP inhibit VC1:IIC2 in complex with Gαs and FS with Kᵢ values in the 10 nM range (Mou et al., 2006). Crystallographic studies showed that the TNP-group binds to the same hydrophobic pocket as the MANT-group. Like MANT-nucleotides, TNP-nucleotides are fluorescent and sensitive to changes in hydrophobicity (Hiratsuka, 2003). Using the bacterial AC toxin from Bordetella pertussis, CyaA, as a model system, we showed that MANT-nucleotides and TNP-nucleotides complement each other in the exploration of activation-dependent conformational changes in ACs (Göttle et al., 2007).

Therefore, the aims of the present study were to examine the interactions of VC1:IIC2 with TNP-nucleotides using fluorescence spectroscopy and to characterize the interactions of various ACs and sGC with TNP-nucleotides. Moreover, we examined the interaction of TNP-nucleotides with G-proteins to obtain some information on selectivity of TNP-nucleotides for other relevant nucleotide-binding proteins involved in signal transduction. Previous studies from our laboratory showed that MANT-nucleotides bind to G-proteins with considerably lower affinity than to ACs (Gille and Seifert, 2003a,b).

Materials and Methods

Materials. VC1, IIC2, and VIIC were purified as described previously (Tesmer et al., 2002). The expression construct for glutathione S-transferase (GST)-human sAC was generated by cloning the coding region of human sAC (arginine acid residues 1–470) into a pGEX-KG vector, in which a TEV protease recognition site was introduced before the start of the human sAC sequence. The plasmid was transformed into the expression strain Escherichia coli BL-21(DE3)-pLys and grown in Luria-Bertani medium containing ampicillin (50 μg/ml) until OD = 0.5. Thereafter, protein expression was induced with 30 μM isopropyl β-D-thiogalactopyranoside at 25°C for an additional 15 to 18 h. The harvested cells were suspended and sonicated in lysis buffer (50 mM Tris/HCl, pH 8.0, 50 mM NaCl, 5 mM dithiothreitol, and protease inhibitors). The crude cell lysate was centrifuged at 18,000g for 1 h at 4°C, and the supernatant fluid was loaded onto a 5-ml glutathione-Sepharose 4B resin column (GE Healthcare, Waukesha, WI). The GST-sAC protein was eluted from the resin with the aforementioned buffer containing 10 mM reduced glutathione (Sigma-Aldrich, St. Louis, MO). The pooled eluted fractions were digested overnight with TEV protease at 4°C. The TEV-digested protein was passed through a column with 1 ml of glutathione-Sepharose 4B resin and 0.5 ml of Talon resin (Clontech, Mountain View, CA) to remove the undigested protein, the GST tag, and TEV protease. Human sAC was dialyzed against a buffer consisting of 50 mM Tris/HCl, pH 7.5, 30 mM NaCl, 5 mM 2-mercaptoethanol, and protease inhibitors. The dialysate was applied onto a 30-ml Fast-Flow Q-Sepharose (GE Healthcare) and eluted with a 0 to 1 M NaCl gradient using an AKTA FPLC system (GE Healthcare). Purified human sAC protein was further passed through sizing columns to remove any remaining contaminants. The purity of the human sAC protein was >95% as determined by SDS polyacrylamide electrophoresis on a 4 to 20% (mass/vol) Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA) and subsequent Coomassie Blue staining.

Baculoviruses encoding ACs I, II, and V were donated by Drs. A. G. Gilman and R. K. Sunahara (University of Texas Southwestern Medical Center, Dallas, TX). Purified sGC was from Alexis (Lausen, Switzerland). Construction and expression of β₂AR-Gαs, and PPR-Gαi, fusion proteins were described previously (Seifert et al., 1998; Wenzel-Seifert et al., 1999; Liu et al., 2001). S9 cells were obtained from the American Type Cell Culture Collection (Manassas, VA). TNP-ATP, TNP-ADP, and TNP-AMP were purchased from Invitrogen (Karlsruhe, Germany). TNP-GTP, TNP-GDP, TNP-UTP, and TNP-CPT were obtained from Jena Bioscience (Jena, Germany). DMB-FS was obtained from Calbiochem (La Jolla, CA). FS, 2′,5′-dideoxy-3′-ATP, myokinase, monocyclohexylammonium phosphoenolpyruvate, and pyruvate kinase were obtained from Sigma-Aldrich. [α-32P]ATP (3000 Ci/mmol), [α-32P]GTP (3000 Ci/mmol), and [γ-32P]GTP (600 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Sources of cell culture reagents and other materials have been described previously (Wenzel-Seifert et al., 1999; Gille and Seifert, 2003a; Gille et al., 2004; Mou et al., 2006).

Cell Culture and Membrane Preparation. Cell culture, expression, and membrane preparation were carried out as described previously (Wenzel-Seifert et al., 1999; Gille et al., 2004). In brief, S9 cells were cultured in SP 900 II medium (Invitrogen) with 5% (v/v) fetal bovine serum and 0.1 mg/ml gentamycin. S9 cells were infected with 1:100 dilutions of baculoviruses encoding different ACs.
or GPCR-Gα fusions and cultured for 48 h. Cells were then harvested by centrifugation for 10 min (1000g at 4°C). Pellets were resuspended in lysis buffer (10 mM Tris/HCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml benzamide, pH 7.4) and disrupted with 20 strokes using a Dounce homogenizer. The resultant suspension was centrifuged for 5 min (500g at 4°C) for sedimentation of nuclei. The supernatant fluid containing the cell membranes was transferred to 30-ml tubes and centrifuged for 20 min (30,000g at 4°C). The resultant pellet was then collected and resuspended in storage buffer containing 75 mM Tris/HCl, 12.5 mM MgCl₂, and 1 mM EDTA, pH 7.4. Protein concentration for each membrane was measured using a Bio-Rad DC protein assay kit. Membrane aliquots (0.5–1 ml each) were stored at −80°C.

**AC and GC Activity Assay.** The AC activity assay with VC1: IIC2 was performed as described previously (Mou et al., 2005, 2006). Assay tubes contained 7 nM VCl and 35 nM IIC2. TNP-nucleotides (final concentration, 1 nM–100 μM) were added. After a preincubation for 2 min at 30°C, 20 μl of reaction mixture containing (final) 100 μM CAMP, 40 μM ATP, 10 mM MnCl₂, 100 mM KCl, 25 mM HEPES/NaOH, pH 7.4, and 1.0–1.5 μCi/tube [³²P]ATP were added to assay tubes. After incubation for 1 h at 30°C, reactions were terminated by adding 20 μl of 2 N HCl. Denatured protein was sedimented by a 1-min centrifugation at 25°C and 15,000g followed by application of 65 μl of the supernatant fluid onto disposable alumina columns containing 1.3-g neutral alumina (Sigma A-1522, super I, WN-6; Sigma-Aldrich). To separate [³²P]cAMP from [³²P]ATP, 4 ml of 0.1 M ammonium acetate was added for elution of [³²P]CAMP. Recovery of [³²P]CAMP was approximately 80% as assessed with [³²P]HCl·AMP set as standard. [³²P]CAMP was determined by liquid scintillation counting using Ecolore scintillation cocktail. AC assays with membranes from Sf9 cells expressing ACs I, II, and V were conducted as described previously (Gille et al., 2004). Membranes were first centrifuged for 15 min (15,000g at 4°C), and the pellets were resuspended in storage buffer. Each assay tube contained membranes expressing an AC isofrom (15–20 μg of protein/tube), 10 μM GTP-S, 40 μM ATP, 100 μM FS, 1 mM MnCl₂ and 100 μM CaCl₂. Reactions were conducted for 20 min at 30°C. Reaction mixtures for sAC contained 200 ng of purified sAC, 50 mM Tris/HCl, pH 7.4, 5 mM MnCl₂, 10 mM CaCl₂, 40 μM ATP, 1.0–1.5 μCi [³²P]ATP, and 100 μM CaCl₂. In some experiments, we assessed the effect of an NTP-regenerating system containing 100 μM ATP, 1.0–1.5 μCi [³²P]ATP, 100 μM CAMP, 100 μM sodium nitroprusside, and TNP-nucleotides (final concentration, 1 nM–100 μM). [³²P]GMP was separated from [³²P]GTP as described for the [³²P]CAMP/[³²P]ATP pair.

**Fluorescence Spectroscopy.** All experiments were carried out using a Cary Eclipse fluorescence spectrophotometer at 25°C (Varian Inc., Walnut Creek, CA) as described previously (Mou et al., 2005, 2006; Göttele et al., 2007; Pinto et al., 2009). In brief, measurements were performed using a quartz fluorescence microcuvette (Hellma, Plainview, NY) in a final assay volume of 150 μl. Steady-state emission spectra were recorded at low speed with λex = 405 nm (λem = 500–600 nm). Reaction mixtures contained 100 mM KCl and 10 mM MnCl₂ in 25 mM HEPES/NaOH, pH 7.4, followed by sequential addition of TNP-nucleotides (final concentration, 5 μM), 4 μM VCl plus 25 μM IIC2, and 100 μM final DMB-FS (water-soluble analog of FS). In contrast to MANT-nucleotides, in TNP-nucleotides, the fluorescent TNP-group does not isomerize between the 2′- and 3′-O-ribosyl position since the TNP substituent is linked to both the 2′- and 3′-O-ribosyl position simultaneously. Accordingly, TNP-nucleotides are more rigid molecules than MANT-nucleotides (Hiratsuka, 1983, 2003; Jameson and Eccleston, 1997). Another difference between MANT-nucleotides and TNP-nucleotides is the fact that the latter nucleotides exhibit a much lower basal fluorescence than the former nucleotides, increasing the potential signal-to-noise ratio in fluorescence studies (Göttele et al., 2007). Moreover, TNP-nucleotides are excited in the visible light range (λem, 405 nm), whereas MANT-nucleotides are excited in the UV light range (λem, 350 nm). Thus, fluorescence studies with TNP-nucleotides are less sensitive to interference with substances absorbing UV light. In MANT-nucleotides, the fluorescent MANT-group can spontaneously isomerize between the 2′- and 3′-O-ribosyl position at physiological pH (Hiratsuka, 1983; Jameson and Eccleston, 1997). Accordingly, MANT-nucleotides are not clearly defined at any given time.

**GTPase Assay.** Steady-state GTPase activity was determined as described previously (Seifert et al., 1998; Wenzel-Seifert et al., 1999; Liu et al., 2001). In brief, membranes from Sf9 cells expressing βAR-Gαs and FPR-Gαs fusion proteins were thawed and centrifuged for 15 min (15,000g at 4°C) to eliminate any endogenous nucleotides. Pellets were resuspended in 10 mM Tris/HCl, pH 7.4. Assay tubes contained 10 μg of protein/tube, 1 mM adenylyl imidodiphosphate, 100 nM unlabeled GTP, 100 μM ATP, 1 mM MgCl₂, 100 mM EDTA, 0.2% (mass/vol) bovine serum albumin, 5 mM creatine phosphate, and 40 μg of creatine kinase in 50 mM Tris/HCl, pH 7.4, and increasing concentrations of TNP-ATP or TNP-GTP ranging from 1 μM to 1 mM and 10 μM isoproterenol (agonist for βAR-Gαs) or 10 μM N-formyl-l-methionyl-l-leucyl-l-phenylalanine (agonist for FPR-Gαs) to fully activate GPCRs and, consequently, GTP hydrolysis by G-proteins. Assay tubes with the reaction mixtures were incubated for 3 min at 25°C following addition of 20 μl of [³²P]GTP (0.2 μCi/tube). Reactions were carried out for 20 min at 25°C and terminated by adding 900 μl of slurry (5% mass/vol activated charcoal and 50 mM NaH₂PO₄, pH 2.0). Reaction mixtures were centrifuged for 15 min (15,000g at room temperature). Seven hundred microliters of supernatant fluid were removed from assay tubes, and [³²P]GTP was determined by liquid scintillation counting. In the presence of 1 mM unlabeled GTP, nonenzymatic degradation of [³²P]GTP was determined and was <1% of the total amount of radioactivity added.

**Molecular Modeling.** Docking calculations were carried out using the GOLD 3.0.1 docking software. As starting point, we used the crystal structure of VC1:IIC2·FS·Gαs-GTP·S in complex with TNP-ATP and two Mn²⁺ ions (Protein Data Bank ID: 1GVD) (Mou et al., 2006). The bound ligand, TNP-ATP, was used as reference for determination of the binding of TNP-GTP. All atoms and their associated residues within 5 Å of each ligand atom were used to define the catalytic site. The information on ligand hydrogen-bonding interactions and conformations was encoded into the corresponding genetic algorithm (GA) of GOLD, using the default GOLD 3.0.1 GA parameters. The Goldscore and Chemscore scoring functions were used to rank binding poses. Exogenous TNP-ATP was aligned to the bound TNP-ATP conformation in the VC1:IIC2 crystal structure. The docked conformation of the optimally aligned TNP-ATP model mimicked the interactions observed in the crystal structure. Thus, the rigid docking procedure reproduced actual ligand/enzyme interactions. The molecular surface of VC1:IIC2 was calculated with the PYMOL software.

**Data Analysis.** All inhibition curves were analyzed by nonlinear regression using Prism 4.0 software (GraphPad, San Diego, CA). Fluorescence spectra were analyzed using the spectrum package of the Cary Eclipse software. For generation of graphs shown in Figs. 1 and 2, data were imported into the Prism software.

**Results**

**Inhibition of the Catalytic Activity of VC1:IIC2 by TNP-Nucleotides.** VC1:IIC2 possesses a broad specificity for purine and pyrimidine nucleotides as is reflected by the fact that the fully activated enzyme, i.e., VC1:IIC2 in the

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**Adenylyl and Guanylyl Cyclase Inhibition by TNP-Nucleotides**

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**References**

[1] Mou, S., Gille, M., Göttele, M., Seifert, A., and Eccleston, P. (2004). Membranes were first centrifuged for 15 min (15,000g at 4°C), and the pellets were resuspended in storage buffer. Each assay tube contained membranes expressing an AC isofrom (15–20 μg of protein/tube), 10 μM GTP-S, 40 μM ATP, 100 μM FS, 1 mM MnCl₂ and 100 μM CaCl₂. Reactions were conducted for 20 min at 30°C. Reaction mixtures for sAC contained 200 ng of purified sAC, 50 mM Tris/HCl, pH 7.4, 5 mM MnCl₂, 10 mM CaCl₂, 40 μM ATP, 1.0–1.5 μCi [³²P]ATP, and 100 μM CaCl₂. In some experiments, we assessed...
presence of FS and $G_{\text{sat}}$-GTP-$\text{S}$, was inhibited with similar potency by TNP-ATP, TNP-GTP, and TNP-UPF, followed by TNP-CTP (Mou et al., 2006). In the presence of FS alone, TNP-ATP, TNP-UTP, and TNP-CTP were about equally potent VC1:IIC2 inhibitors, followed by TNP-GTP. The omission of $G_{\text{sat}}$-GTP-$\text{S}$ had only minor effects on overall TNP-nucleotide potency (Mou et al., 2006).

For fluorescence studies with TNP-nucleotides, it was important to determine whether TNP-nucleotides can also bind to VC1:IIC2 in the absence of activators (Mou et al., 2005, 2006). In fact, in the absence of the activators FS and $G_{\text{sat}}$-GTP-$\text{S}$, TNP-NDPs and TNP-NTPs inhibited the catalytic activity of VC1:IIC2, with potencies that differed from each other by <5-fold (Table 1). However, compared to the conditions with FS, the potencies of TNP-NTPs were reduced by up to 150-fold in the absence of FS (Mou et al., 2006).

**Fluorescence Studies with VC1:IIC2 and TNP-Nucleotides.** In agreement with previous data (Hiratsuka, 2003; Göttle et al., 2007), TNP-nucleotides exhibited only small fluorescence signals in the absence of VC1:IIC2 (Fig. 1). However, VC1:IIC2 largely increased these fluorescence signals with TNP-ATP, TNP-ADP, TNP-GTP, TNP-GDP, TNP-CTP, and TNP-UTP, compatible with the assumption that TNP-nucleotides bind to the enzyme and that the TNP-group inserts into the hydrophobic pocket between VC1 and IIC2 that is also occupied by MANT-nucleotides (Mou et al., 2005, 2006). In the absence of VC1:IIC2, TNP-nucleotides exhibited an emission maximum of 545 nm (Table 2). The addition of VC1:IIC2 shifted the emission maximum toward shorter wavelengths, a process referred to as blue-shift. The blue-shift exhibited nucleotide-specific differences, with TNP-GTP showing the largest shift. The nonfluorescent ATP analog, 2',5'-dideoxy-3'-ATP, binds to the catalytic site of VC1:IIC2 with high affinity (Tesmer et al., 2000; Gille et al., 2004). To assess the specificity of the fluorescence increases of TNP-nucleotides upon addition of VC1:IIC2, we examined the effect of 2',5'-dideoxy-3'-ATP. In fact, 2',5'-dideoxy-3'-ATP (200 μM) strongly reduced the fluorescence increase of TNP-ATP (5 μM) caused by the addition of VC1:IIC2 (Fig. 2). These data show that...
**TABLE 1**

Inhibition of VC1:IIC2 by TNP-nucleotides

<table>
<thead>
<tr>
<th>TNP-Nucleotide</th>
<th>(K_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNP-AMP</td>
<td>&gt; 100,000</td>
</tr>
<tr>
<td>TNP-ADP</td>
<td>11,000 ± 5900</td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>3300 ± 1400</td>
</tr>
<tr>
<td>TNP-GDP</td>
<td>15,000 ± 1000</td>
</tr>
<tr>
<td>TNP-GTP</td>
<td>6400 ± 2000</td>
</tr>
<tr>
<td>TNP-UTP</td>
<td>14,000 ± 1200</td>
</tr>
<tr>
<td>TNP-CTP</td>
<td>8600 ± 1100</td>
</tr>
</tbody>
</table>

TNP-nucleotides and 2',5'-dideoxy-3'-ATP compete for binding to the catalytic site of VC1:IIC2.

The addition of the water-soluble FS-analog, DMB-FS (Pinto et al., 2008, 2009), to cuvettes containing TNP-nucleotides and VC1:IIC2 decreased the fluorescence signals of TNP-nucleotides (Fig. 1). However, TNP-nucleotides exhibited large differences in their sensitivity toward reduction of fluorescence upon addition of DMB-FS. Specifically, with TNP-ATP, TNP-CTP, and TNP-UTP, large decreases in fluorescence were observed, whereas the decrease was moderate with TNP-ADP and only marginal with TNP-GTP and TNP-GDP.

**Molecular Modeling of the Interaction of TNP-GTP and TNP-ATP with VC1:IIC2.** To obtain direct evidence for distinct interactions of TNP-nucleotides with VC1:IIC2, we undertook substantial efforts to produce a crystal structure of VC1:IIC2 in complex with \(G_{mATP}\):GTP, Mn\(^{2+}\) and TNP-GTP. Unfortunately, our efforts have been unsuccessful for unknown reasons (data not shown). Therefore, as an alternative approach, we conducted molecular modeling studies. Using the crystal structure of VC1:IIC2 in complex with TNP-ATP as template (Mou et al., 2006), TNP-GTP was docked into the catalytic site of the enzyme (Fig. 3). Overall, TNP-ATP and TNP-GTP adopt similar positions in the catalytic site, and both nucleotides interact with the amino acids Asn1025 and Lys1065 of IIC2 (Fig. 3A) (Mou et al., 2006). Figure 3A also shows the insertion of the TNP-group into the hydrophobic pocket between IIC2 and VC1, providing an explanation for the fluorescence increase of TNP-nucleotides upon binding to the enzyme (Fig. 1). Although TNP-ATP and TNP-GTP bind to VC1:IIC2 similarly, there are also subtle differences. In particular, the N2-atom of guanine forms a hydrogen bond with Asp1018, resulting in slightly different orientations of both the triphosphate chain and the TNP-group (Fig. 3B).

**Inhibition of the Catalytic Activity of AC Isoforms and sGC by TNP-Nucleotides.** ACV inhibitors may be valuable drugs for the treatment of cardiovascular diseases, specifically chronic heart failure (Iwatsubo et al., 2004; Rottlaender et al., 2007; Yan et al., 2007; Götte et al., 2009), and sAC inhibitors may represent a novel class of male contraceptives (Schlicker et al., 2008). Therefore, we systematically studied the potencies of TNP-nucleotides for inhibition of ACs I, II, and V and sAC (Table 3). We also included sGC into our studies because this enzyme is structurally related to membranous ACs (Sunahara et al., 1998) and exhibits broad-base specificity as well (Gille et al., 2004). Moreover, sGC plays an important role in the regulation of numerous cardiovascular and neuronal processes, but there is a paucity of potent and selective substrate-binding site-based sGC inhibitors (Derbyshire and Marletta, 2009; Schmidt et al., 2009).

TNP-nucleotides inhibited ACI in the order of potency TNP-UTP ~ TNP-ATP ~ TNP-GTP ~ TNP-CTP > TNP-ADP > TNP-GDP ≫ TNP-AMP (Table 3). ACII was less sensitive to inhibition by TNP-nucleotides than ACI. These data are in agreement with the results obtained with MANT-nucleotides (Gille et al., 2004; Götte et al., 2009). However, we also noted that the order of potency of TNP-nucleotides for inhibition of ACs I and II was different. Specifically, the order of potency for ACII was TNP-UTP > TNP-ATP ~ TNP-CTP > TNP-GTP ≫ TNP-ADP > TNP-GDP > TNP-AMP (Table 3). Like ACI, ACV showed high sensitivity to inhibition by TNP-nucleotides. These data are again in agreement with the results obtained with MANT-nucleotides (Gille et al., 2004; Götte et al., 2009). However, the inhibitor profiles were different for both AC isoforms. Specifically, TNP-nucleotides inhibited ACV in the order of potency: TNP-ATP > TNP-UTP > TNP-GTP ~ TNP-CTP > TNP-ADP > TNP-GDP > TNP-AMP (Table 3). The substantial loss of potency of TNP-ADP and TNP-AMP relative to TNP-ATP, as well as TNP-GDP relative to TNP-GTP, in all mACs studied is explained by the loss of ionic interactions between the β- and γ-phosphates with Lys1065 of IIC2 and metal ion B (Mou et al., 2006).

In accordance with the data obtained for MANT-nucleotides (Gille et al., 2004), sAC was considerably less sensitive to inhibition by TNP-nucleotides than mACs. Among the compounds examined, TNP-ATP was the most potent sAC inhibitor, but the nucleotide was 7- to 200-fold less potent than at ACs I, II, and V (Table 3). These data show that the structure/activity relationships for nucleotide-based inhibitors of mACs and sAC are quite different and that it will be very challenging to obtain highly potent AC inhibitors with selectivity relative to mACs.

In marked contrast, sGC was very sensitive to inhibition by TNP-nucleotides, with ACV and sGC showing substantial similarities in the inhibition profile (Table 3). TNP-ATP was a slightly more potent sGC inhibitor than TNP-GTP. Notably, the pyrimidine nucleotides TNP-UTP and TNP-CTP were just 3- to 5-fold less potent sGC inhibitors than the
purine nucleotides. To the best of our knowledge, TNP-ATP is the most potent sGC inhibitor reported so far (Derbyshire and Marletta, 2009; Schmidt et al., 2009). Cell membrane-permeable TNP-nucleotides may become very valuable tools for the analysis of sGC in cardiovascular and neuronal functions. Moreover, TNP-nucleotides may be useful ligands for crystallizing sGC, of which the goal has not yet been accomplished.

**Inhibition of the Catalytic Activity of G\textsubscript{\alpha\textprime} and G\textsubscript{\gamma}\textprime Proteins by TNP-Nucleotides.** The steady-state GTPase assay with GPCR-G\textsubscript{\alpha} fusion proteins is a very well suited assay for the determination of G\textsubscript{\alpha\textprime} and G\textsubscript{\gamma}\textprime-protein affinities for nucleotides (Seifert et al., 1999; Gille et al., 2002, 2005; Gille and Seifert, 2003b). In a previous study we showed that MANT-guanosine 5’-[\gamma\textprime]-thio(triphosphate and MANT-guanosine 5’-[\beta\gamma\textprime]-imido(triphosphate bind to G\textsubscript{\gamma}\textprime} and G\textsubscript{\alpha\textprime}-proteins with low-affinity, i.e., in the 0.2 to 1.0 \mu\text{M} and 1 to 6 \mu\text{M} range, respectively (Gille and Seifert, 2003b). This low-affinity interaction of MANT-nucleotides is explained by the tight guanine nucleotide-binding pocket of G-protein \(\alpha\)-subunits, leaving little space to accommodate bulky substituents at the 2’- and 3’-O-ribosyl position (Seifert and Gille, 2003b). Accordingly, we expected the conformationally constrained TNP-nucleotides also to bind to G-proteins with low affinity. Indeed, TNP-GTP inhibited agonist-stimulated steady-state GTP hydrolysis of various \(\beta\alpha\textprime}-G\textsubscript{\alpha\textprime} and FPR-G\textsubscript{\alpha\textprime} fusion proteins only with \(K_i\) values in the 10 to 30 \mu\text{M} range (Table 4). Considering then that TNP-GTP inhibits holo-ACs and sGC with \(K_i\) values in the 10 to 200 nM range (Table 3), it becomes clear that TNP-GTP exhibits an exquisite selectivity for nucleotidyl cyclases compared with G-proteins. TNP-ATP binds to G\textsubscript{\alpha\textprime} and G\textsubscript{\gamma}\textprime-proteins with even lower affinity than TNP-GTP, i.e., with \(K_i\) values \(>50\) to 100 \mu\text{M}. Thus, TNP-ATP exhibits \(>1000\)-fold selectivity for some ACs relative to G-proteins. The low affinity of G-proteins for TNP-GTP also implies that this nucleotide is very unlikely to be useful as fluorescent probe for monitoring conformational changes in G-proteins.

**Discussion**

**TNP-Nucleotides as Experimental Tools.** TNP-nucleotides are broadly used as fluorescence probes for many nucleotide-binding proteins, including receptors, enzymes, and structural proteins (Hiratsuka, 2003). To the best of our knowledge, mACs exhibit the highest affinity for TNP-nucleotides among all target proteins studied so far (Table 3). Most proteins bind TNP-nucleotides with affinities in the micromolar range (Tables 1 and 3) (Mou et al., 2006). Moreover, TNP-nucleotides possess an excellent selectivity for mACs relative to G-proteins (Table 4). This striking selectivity of TNP-nucleotides for mACs relative to G-proteins could be exploited in electrophysiological signal transduction studies with intact cells in which nucleotides are introduced as AC inhibitors into the cytosol via the patch pipette (Rottlaender et al., 2007).

**Broad-Base Specificity of ACs and sGC.** Our present study corroborates the unexpected notion that mACs exhibit a broad-base specificity and high conformational flexibility (Gille and Seifert, 2003; Gille et al., 2004; Mou et al., 2005, 2006; Göttle et al., 2009). This notion can be extended to structurally unrelated bacterial AC toxins (Göttle et al., 2007; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3). Thus, despite the substantial structural differences between all of the nucleotidyl cyclases studied so far (Sunahara et al., 1998; Sunahara and Taussig, 2002; Ahuja et al., 2009; Taha et al., 2009) and, as shown here, to sGC and sAC (Table 3).
TABLE 3  
Inhibition of various AC isoforms and sGC by TNP-nucleotides

<table>
<thead>
<tr>
<th>TNP-Nucleotide</th>
<th>$K_i$ ($\pm$ S.D.)</th>
<th>$K_i$ ($\pm$ S.D.)</th>
<th>$K_i$ ($\pm$ S.D.)</th>
<th>$K_i$ ($\pm$ S.D.)</th>
<th>$K_i$ ($\pm$ S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNP-AMP</td>
<td>4800 ± 1000</td>
<td>7400 ± 520</td>
<td>4800 ± 1200</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>TNP-ADP</td>
<td>140 ± 17</td>
<td>110 ± 180</td>
<td>370 ± 45</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>9.0 ± 4.1</td>
<td>99 ± 2.7</td>
<td>3.7 ± 1.0</td>
<td>710 ± 82</td>
<td>7.3 ± 1.7</td>
</tr>
<tr>
<td>TNP-GDP</td>
<td>410 ± 76</td>
<td>3400 ± 1300</td>
<td>1200 ± 280</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>TNP-GTP</td>
<td>23 ± 2.2</td>
<td>220 ± 30</td>
<td>27 ± 4.3</td>
<td>7200 ± 280</td>
<td>8.6 ± 2.6</td>
</tr>
<tr>
<td>TNP-UTP</td>
<td>7.1 ± 2.3</td>
<td>24 ± 8.4</td>
<td>15 ± 2.2</td>
<td>3100 ± 50</td>
<td>33 ± 6.9</td>
</tr>
<tr>
<td>TNP-CTP</td>
<td>24 ± 2.9</td>
<td>110 ± 35</td>
<td>31 ± 3.9</td>
<td>5100 ± 480</td>
<td>27 ± 0.9</td>
</tr>
</tbody>
</table>

N.D., not determined.

TABLE 4  
Affinities of Gα- and Gβ-proteins for TNP-ATP and TNP-GTP

<table>
<thead>
<tr>
<th>Construct</th>
<th>$K_i$ ($\pm$ S.D.)</th>
<th>$K_i$ ($\pm$ S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>βAR-G&lt;sub&gt;95&lt;/sub&gt;</td>
<td>53,000 ± 11,000</td>
<td>12,000 ± 1500</td>
</tr>
<tr>
<td>βAR-G&lt;sub&gt;91&lt;/sub&gt;</td>
<td>&gt;100,000</td>
<td>17,000 ± 2100</td>
</tr>
<tr>
<td>FPR-&lt;sub&gt;92&lt;/sub&gt;</td>
<td>&gt;100,000</td>
<td>26,000 ± 3400</td>
</tr>
<tr>
<td>FPR-&lt;sub&gt;92&lt;/sub&gt;</td>
<td>&gt;100,000</td>
<td>12,000 ± 3100</td>
</tr>
<tr>
<td>FPR-&lt;sub&gt;93&lt;/sub&gt;</td>
<td>&gt;100,000</td>
<td>6900 ± 1300</td>
</tr>
<tr>
<td>FPR-&lt;sub&gt;93&lt;/sub&gt;</td>
<td>&gt;100,000</td>
<td>10,000 ± 2200</td>
</tr>
</tbody>
</table>

al., 2004), broad-base specificity is a common and highly conserved theme in this enzyme class. The broad-base specifici-  
ity and conformational flexibility, however, do not imply that all nucleotidyl cyclases exhibit identical pharmacologi-  
cal profiles. In fact, each nucleotidyl cyclase exhibits a unique profile (Table 3). Moreover, the differential effects of  
DMF-S and the differences in blue-shift in our fluorescence  
 studies revealed that purine and pyrimidine nucleotides bind  
to the catalytic site of at least one AC (VCI:IIC2) in slightly  
different manners (Fig. 1, Table 2).

We also would have liked to have conducted fluorescence  
studies with sGC that based on the high affinity of this  
enzyme for TNP-nucleotides are technically feasible. How-  
ever, a drawback of the fluorescence studies is the fact that  
high protein concentrations (in the 5–25  
 ever, a drawback of the fluorescence studies is the fact that  
high protein concentrations (in the 5–25 μM range) are re-  
quired (see Materials and Methods). Unfortunately, it is not  
yet possible to obtain such large amounts of sGC. Conversely,  
large amounts of recombinant sAC can be readily obtained  
(see Materials and Methods), but the affinity of sAC for  
TNP-nucleotides is too low for fluorescence studies (Table 3).

Whereas MANT-nucleotides are more potent inhibitors of  
VCI:IIC2 than of holo-ACs I and V (Gille et al., 2004), the  
opposite is true of TNP-nucleotides (Tables 1 and 3) (Mou et  
al., 2006). Thus, the structural constraints in TNP-nucleo-  
tides caused by the dual and nonisomerizing 2'- and 3'-O-  
ribosyl modification compared with the isomerizing 2'- and  
3'-O-ribosyl isomerization in MANT-nucleotides (Jameson and  
Eccleston, 1997; Hiratsuka, 2003) facilitate binding to  
holo-ACs. A major structural difference between VCI:IIC2 and  
holo-ACs is that the former enzyme is completely devoid of  
the transmembrane domains (Sunahara and Taussig,  
2002). These data indicate that the transmembrane domains,  
perhaps by modulation of the mobility of the catalytic do-  
main, differentially regulate inhibitor affinities of holo-ACs.

The high affinity of ACs I, II, and V and sGC for TNP-UTP  
and TNP-CTP is quite remarkable. These data raise the  
improving question whether UTP and CTP can also be sub-  
strates for ACs. In support of such a possibility is the fact  
that both 3',5'-cyclic CMP and 3',5'-cyclic UMP do, indeed,  
occur in mammalian tissues as shown by sensitive mass spectrometry techniques (Newton et al., 1984, 1986). More-  
ever, in experiments with [α-32P]UTP as substrate,  
[32P]cUMP was identified as a bona fide product of sGC (Gille  
et al., 2004), but direct structural confirmation of cUMP  
production by sGC with mass spectrometry techniques is still  
missing. The structural identity of the putative mammalian  
cytidyl cyclase is elusive as well (Newton et al., 1990).

Comparison of TNP-Nucleotides and MANT-Nucleo-  
tides as Fluorescence Probes. The crystal structures of  
VCI:IIC2 with MANT-GTP, MANT-ATP, and TNP-ATP  
show that, in all three cases, the fluorescent group, be it a  
MANT-group or a TNP-group, resides in a hydrophobic  
 pocket between both sub-  
units can already form in the absence of AC activators. In  
contrast, MANT-nucleotides and TNP-nucleotides  
respond with an increase in fluorescence upon exposure to a  
hydrophobic environment (Jameson and Eccleston, 1997;  
Hiratsuka, 2003), one may have predicted to observe similar  
fluorescence changes with both classes of nucleotides upon  
interaction with VCI:IIC2. However, this was clearly not the  
case. Most strikingly, even in the absence of DMB-FS, VCI:  
IIC2 caused large fluorescence increases with TNP-nucleo- 
tides and blue-shifts of the emission maximum (Fig. 1, Table 2), indicating that the hydrophobic pocket between both sub-  
units can already form in the absence of AC activators. In  
contrast, MANT-nucleotides are quite ineffective at inserting  
into the hydrophobic pocket of VCI:IIC2 in the absence of  
activators (Mou et al., 2005, 2006). Whereas FS translocates  
the MANT-group into a more hydrophobic environment, res-  
sulting in fluorescence increases (Mou et al., 2005, 2006;  
Pinto et al., 2009), FS translocates the TNP-group into a  
more hydrophilic environment, resulting in fluorescence de-  
creases. Intriguingly, both among MANT-nucleotides (Mou et  
al., 2006) and TNP-nucleotides (Fig. 1, Table 2), there are  
substantial base-specific differences in fluorescence proper- 
ties. Those differences were not predicted either by crystal- 
lographic studies (Mou et al., 2006) or molecular modeling  
(Fig. 3). Thus, fluorescence spectroscopy is much more sensitive than crystallography and molecular modeling.
at revealing very subtle conformational differences among similar ligands bound to mAC. These data corroborate the concept of conformational flexibility of ACs.

There are also some interesting similarities in the fluorescence properties of MANT-nucleotides and TNP-nucleotides at VC1:IIC2 compared with the bacterial AC toxin CyaA that is activated by calmodulin. Specifically, with MANT-nucleotides, there is an increase in direct fluorescence and fluorescence resonance energy transfer upon interaction of CyaA with calmodulin, whereas with TNP-nucleotides, there is a calmodulin-dependent decrease in fluorescence (Göttle et al., 2007). The mAC activator FS revealed similar patterns with MANT- and TNP-nucleotides at VC1:IIC2 (Mou et al., 2005, 2006; Pinto et al., 2009) (Fig. 2). Moreover, the fluorescence decreases with TNP-nucleotides at CyaA are, like with VC1:IIC2, base-dependent. Strikingly, the activation-dependent decreases in fluorescence at CyaA and VC1:IIC2 with TNP-nucleotides were most prominent for the pyrimidine bases (Göttle et al., 2007). These data indicate that, despite the substantial structural differences between mammalian membranous ACs and the bacterial AC toxin (Sunahara and Tausigg, 2002; Ahuja et al., 2004), there are common and highly conserved themes in activation-dependent conformational changes in both classes of enzymes. Probably, these conserved conformational changes are directly related to the broad-base specificity found in mACs and bacterial AC toxins (Gille et al., 2004, 2005; Mou et al., 2006; Gottle et al., 2007, 2009; Taha et al., 2009). Like mAC and CyaA, edema factor AC toxin from Bacillus anthracis shows activation-dependent fluorescence increases with MANT-nucleotides (Taha et al., 2009). However, the affinity of edema factor for TNP-nucleotides is too low to allow for the conduction of fluorescence studies (data not shown).

**Future Development of mAC Inhibitors.** The development of isofrom-selective AC inhibitors is an important and ambitious long-term goal. In particular, ACV inhibitors are promising drug candidates for the treatment of heart failure and ageing-related diseases (Iwatsubo et al., 2004; Rottlander et al., 2007; Yan et al., 2007; Gottle et al., 2009). The identification of TNP-nucleotides as holo-AC inhibitors is an important step toward the achievement of this goal since TNP-nucleotides surpass the corresponding MANT-nucleotides in terms of potency at ACV by up to 50-fold (Table 3) (Gille et al., 2004; Gottle et al., 2009). MANT-inosine 5’-triphosphate is the most potent ACV inhibitor known so far (Ki, 1 nM) (Gottle et al., 2009). Thus, we predict that TNP-inosine 5’-triphosphate, which is not available to us at the present time, will be an even more potent ACV inhibitor. Whereas TNP-nucleotides do not surpass MANT-nucleotides in terms of AC isofrom selectivity, the 2’,3’-O-riboseyl substitution with a phenyl ring offers numerous opportunities for chemical modifications. Specifically, some of the nitro groups attached to the phenyl ring could be deleted or exchanged against other functional groups. Given the conformational flexibility of the catalytic site of mACs together with the fact that the inhibition profiles of various mACs are not identical, the systematic analysis of the structure/activity relationships of phenyl ring substitutions combined with molecular modeling studies may ultimately yield AC isoform-selective inhibitors.

In a previous study, we showed that MANT-GDP and MANT-ADP are phosphorylated to MANT-GTP and MANT-ATP, respectively, by the NTP-regenerating system consisting of mono(cyclohexyl)ammonium phosphoenol pyruvate, pyruvate kinase, and myokinase (Gille et al., 2004). Likewise, the NTP-regenerating system increased the inhibitory potencies of TNP-AMP and TNP-ADP to the values of TNP-ATP using ACV as model AC (data not shown). These data show that TNP-AMP and TNP-ADP can be readily phosphorylated. This is an important property for intact cell experiments with lipophilic pronucleotide inhibitors that have to be converted to the actual inhibitory compounds (Laux et al., 2004). Thus, the present study provides an important starting point for the development of potent and selective mAC and sGC inhibitors.

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**References**


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