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Differential interactions of G-proteins and adenylyl cyclase with nucleoside 5′-triphosphates, nucleoside 5′-[γ-thio]triphosphates and nucleoside 5′-[β,γ-imido]triphosphates

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AC, adenylyl cyclase
AppNHp, adenosine 5′-[β,γ-imido]triphosphate
ATPαS, adenosine 5′-[α-thio]triphosphate

ABSTRACT

The regulatory G-proteins of adenylyl cyclase (AC), G_i and G_s, are not only activated by GTP and the stable GTP analogs, guanosine 5′-[γ-thio]triphosphate (GTPγS) and guanosine 5′-[β,γ-imido]triphosphate (GppNHp), but also by hypoxanthine, xanthine, uracil and cytidine nucleotides. The latter nucleotides were previously used to analyze distinct active G-protein states. Surprisingly, recent studies have shown that inosine 5′-[γ-thio]triphosphate and uridine 5′-[γ-thio]triphosphate can also inhibit AC directly. Therefore, we systematically compared the interactions of nucleoside 5′-triphosphates (NTPs), nucleoside 5′-[γ-thio]triphosphates (NTPγSs) and nucleoside 5′-[β,γ-imido]triphosphates (NppNHps) with G_i, G_s and AC. NTPγSs exhibited up to 26,000-fold higher affinity for G-proteins than NTPs and NppNHps. NTPγSs were up to 150-fold more potent direct AC inhibitors than NTPs and NppNHps. G-proteins exhibited striking preference for guanine nucleotides compared to other purine and pyrimidine nucleotides, whereas base-selectivity of various ACs, particularly the purified catalytic subunits C1·C2, was rather poor. GTP, GTPγS and GppNHp exhibited much higher selectivity for G-proteins relative to AC than all other purine and pyrimidine nucleotides. We have energetically characterized the interactions of purine and pyrimidine nucleotides with AC in silico, constructing pharmacophore models that correlate well with experimental affinities and have elucidated specific amino acid residues with greatest influence on nucleotide binding. Collectively, both G-proteins and ACs bind purine and pyrimidine nucleotides, with G-proteins showing much higher base-selectivity than AC. Thus, direct inhibitory effects of nucleotides on AC should be understood and considered when probing distinct active G-protein states with non-guanine nucleotides.

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ATPγS, adenosine 5′-[γ-thio]triphosphate
β_2AR-$G_{s\alpha L}$, fusion protein consisting of the β_2-adrenoceptor and the long splice variant of the stimulatory G-protein of adenylyl cyclase, $G_{s\alpha}$
CTPγS, cytidine 5′-[γ-thio]triphosphate
FPR-$G_{i\alpha 2}$, fusion protein consisting of the formyl peptide receptor and the inhibitory G-protein of adenylyl cyclase, $G_{i\alpha 2}$
$G_{i\alpha}$, inhibitory G-protein of AC
$G_{s\alpha}$, stimulatory G-protein of AC
$G_{s\alpha L}$, long splice variant of $G_{s\alpha}$
GppNHp, guanosine 5′-[β,γ-imido]triphosphate
GTPγS, guanosine 5′-[γ-thio]triphosphate
IppNHp, inosine 5′-[β,γ-imido]triphosphate
ITPγS, inosine 5′-[γ-thio]triphosphate
NppNHp, nucleoside 5′-[β,γ-imido]triphosphate
NTPγS, nucleoside 5′-[γ-thio]triphosphate
NTP, nucleoside 5′-triphosphate
UppNHp, uridine 5′-[β,γ-imido]triphosphate
UTPγS, uridine 5′[γ-thio]triphosphate
XppNHp, xanthosine 5′-[β,γ-imido]triphosphate
XTPγS, xanthosine 5′-[γ-thio]triphosphate

1. Introduction

G-proteins act as signal transducers between receptors and effectors [1,2]. G-proteins consist of a α-subunit that contains the nucleotide-binding site and a βγ-complex. It is generally accepted that G-proteins exist in the GTP-bound on-state and the GDP-bound off-state [1–3]. Agonist-occupied receptors promote GDP-dissociation from G_α which constitutes the rate-limiting step of the G-protein cycle. Subsequently, GTP binds to G_α, $G_{s\alpha\text{-GTP}}$ activates AC, and $G_{i\alpha\text{-GTP}}$ inhibits AC. G_α exhibits GTPase activity that acts as an off-switch to generate $G_{\alpha\text{-GDP}}$.

Early [4–6] and more recent studies [7–10] showed that in addition to GTP, other NTPs, i.e. the purine nucleotides ITP and XTP, and the pyrimidine nucleotides, UTP and CTP, are capable of supporting receptor-mediated G-protein activation. NTPs exhibit different efficacies in this respect, giving rise to the hypothesis that G_α can exist in distinct active states, depending on the nucleotide bound [9,10]. Since the hydrolysis-resistant guanine nucleotides GTPγS and GppNHp are most valuable experimental tools for studying G-protein activation [1,11], a logical extension of our research was to examine NTPγSs and NppNHps with bases other than guanine as tools to further probe the concept of multiple active G-protein states. Most unexpectedly, however, during the course of those studies, we observed that the purine nucleotide ITPγS and the pyrimidine nucleotide UTPγS exhibited biphasic effects on AC activity in S49 wild-type lymphoma cell membranes and $G_{s\alpha}$-deficient S49 *cyc*$^-$ lymphoma cell membranes. Specifically, ITPγS and UTPγS exhibited high-potency stimulatory and inhibitory effects ($EC_{50}/IC_{50} \sim 0.2$–0.3 μM) on AC activity that were compatible with the activation of $G_{s\alpha}$ and $G_{i\alpha}$, respectively, in the two membrane systems [12]. In addition, ITPγS and UTPγS exhibited low-potency inhibitory effects (IC_{50} ~20–70 μM) in both systems that were due to direct AC inhibition [12]. Based on these dual effects of ITPγS and UTPγS, the aim of the present study was to provide a systematic analysis of the interactions of NTPs, NTPγSs and NppNHps with G-proteins and AC.

2. Methods

2.1. Materials

ITPγS, XTPγS, UTPγS and CTPγS were prepared by nucleoside diphosphokinase-catalyzed transthiophosphorylation of the respective nucleoside 5′-diphosphates with adenosine 5′-[γ-thio]triphosphate as thiophosphoryl group donor and purified by Mono Q ion exchange chromatography, resulting in product purity >98% [13]. The catalytic AC subunits C1(AC5) and C2(AC2) were purified as described [14]. The preparation of baculoviruses encoding β_2AR-$G_{s\alpha L}$ and FPR-$G_{i\alpha 2}$ fusion proteins was described previously [15,16]. [γ-^{32}P]GTP (6000 Ci/mmol) and [α-^{32}P]ATP (3000 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). ATP, GTP, GTPγS, GppNHp, UTP and CTP were obtained from Roche (Indianapolis, IN, USA). ITP, XTP, IppNHp, XppNHp and UppNHp were obtained from JenaBioscience (Jena, Germany). Forskolin, (−)-isoproterenol and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine were from Sigma (St. Louis, MO, USA).

2.2. Sf9 cell culture, generation of recombinant baculoviruses and membrane preparation

Sf9 cells were cultured in 250 ml disposable Erlenmeyer flasks at 28 °C under rotation at 125 rpm in SF 900 II medium (Gibco, Carlsbad, CA, USA). Sf9 cells were infected with 1:100 dilutions of high-titer baculovirus stocks encoding for β_2AR-$G_{s\alpha L}$ and FPR-$G_{i\alpha 2}$ fusion proteins and cultured for 48 h. Membranes from infected Sf9 cells or uninfected cells were prepared as described [15]. Membranes were suspended in a buffer consisting of 12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4, at a concentration of ~1–2 mg of protein/ml and stored at −80 °C until use. Immediately prior to GTPase- and AC experiments, membrane aliquots were thawed, centrifuged for 15 min at 4 °C and 15,000 × *g* and resuspended in the above-described buffer to reduce remaining nucleotides as far as possible.

2.3. S49 cyc⁻ lymphoma cell culture and membrane preparation

S49 cyc⁻ lymphoma cells were grown at 37 °C in suspension in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4.5 g/l D-glucose, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/l streptomycin and 10% (v/v) heat-inactivated horse serum in a humidified atmosphere containing 5% (v/v) CO_2. S49 cyc⁻ cells were maintained at a density of 0.2–2.0 × 10^6 cells/ml. DMEM medium was from Cellgro Mediatech (Herndon, VA, USA). All other constituents for the culture of S49 cyc⁻ cells were obtained from Bio Whittaker (Walkersville, MD, USA). S49 cells were disintegrated by nitrogen cavitation at 4 °C, 7000 kPa for 30 min in a buffer consisting of 50 mM KH_2PO_4, 100 mM NaCl and 0.5 mM EDTA, pH 7.0. Subsequently, S49 cyc⁻ membranes were prepared as described for Sf9 membranes [15].

2.4. GTPase assay

Determination of GTPase activity in Sf9 membranes was performed as described [17]. Briefly, tubes (80 μl) contained membranes (10 μg of protein per tube), 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 1 mM AppNHp, 5 mM creatine phosphate, 40 μg creatine kinase, 100 nM unlabeled GTP and 0.05% (m/v) bovine serum albumin in 50 mM Tris/HCl, pH 7.4. For experiments with β_2AR-$G_{s\alpha L}$, tubes additionally contained 10 μM (−)-isoproterenol to maximally stimulate GTP hydrolysis. For experiments with FPR-$G_{i\alpha 2}$, tubes additionally contained 10 μM *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine to maximally stimulate GTP hydrolysis. Tubes also contained various nucleotides between 1 nM and 10 mM to inhibit GTP hydrolysis. After 3 min of preincubation at 25 °C, reactions were initiated by adding [γ-^{32}P]GTP (0.2 μCi/tube). Reactions were conducted for 20 min at 25 °C and terminated by adding 900 μl of slurry containing 5% (m/v) activated charcoal in 50 mM NaH_2PO_4 (pH 2.0). Harvesting [^{32}P]P_i in the supernatant fluid of reaction mixtures and scintillation counting were performed as described [17].

2.5. AC assay

The determination of AC activity in Sf9 membranes was performed as described [17]. Briefly, tubes (30 μl) contained membranes (20–40 μg of protein per tube), 5–10 mM $MgCl_2$, 0.4 mM EDTA, 30 mM Tris/HCl, pH 7.4, and nucleotides at various concentrations. In some experiments, 10 mM $MnCl_2$ was used instead of 10 mM $MgCl_2$. Tubes were incubated for 3 min at 37 °C before adding 20 μl of reaction mixture containing (final) [α-^{32}P]ATP (1.0 μCi/tube) plus 40 μM ATP, 2.7 mM mono(cyclohexyl)ammonium phosphoenolpyruvate, 0.125 IU pyruvate kinase, 1 IU myokinase and 0.1 mM cAMP. In experiments with purified catalytic AC subunits, reaction mixtures contained 6 nM C1 plus 30 nM C2 (Mn^{2+} experiments) or 12 nM C1 plus 60 nM C2 (Mg^{2+} experiments). In experiments with S49 cyc⁻ membranes, reaction mixtures contained 100 μM forskolin and 10 mM $MnCl_2$ to maximize AC activity. In addition, $MnCl_2$ at such a high concentration blocks the inhibitory effect of $G_{i\alpha}$ on AC [18–20]. In experiments with membranes from uninfected Sf9 membranes, reaction mixtures contained 100 μM forskolin, 10 mM $MnCl_2$, 10 mM NaF and 10 μM $AlCl_3$ to maximally activate AC and $G_{s\alpha}$. Reactions were conducted for 20 min and terminated by adding 20 μl 2.2N HCl. Separation of [α-^{32}P]ATP from [^{32}P]cAMP was performed as described [17]. The K_m values of ACs in S49 cyc⁻ lymphoma cell membranes and Sf9 insect cell membranes were calculated as described [12,20].

2.6. Molecular modeling

The receptor model was constructed from a crystal structure of C1·C2 with the ligand ATPαS affixed to the nucleotide binding site (PDB entry 1CJK) [21]; the amino acid residue and metal ion numbering used throughout this paper corresponds to the nomenclature used in PDB entry 1CJK. Hydrogen atoms were added according to conventional protonation states (all aspartate and glutamate residues left as anions, and lysine and arginine as cations), and AMBER charges were assigned [22]. All ligands were specified in tetra-anionic form, with partial charges assigned according to the MMFF94 force field [23]. Ligands were then manually docked into the receptor through computational alignment to the originally co-crystal-

lized ATPαS via the MOE program [24]. Fifty alignment runs were performed for each ligand, permitting torsional flexibility and using similarity measures defined by the presence of H-bond donors and acceptors, partial atomic charges, aromaticity, hydrophobicity and volume. Additional distance restraints were imposed at default values to accentuate overlap between the phosphate atoms on the ligand and the template ATPαS. For each ligand, the optimally aligned structure was refined through molecular mechanics minimization (also via MOE), permitting all ligand atoms to relax freely by constraining the receptor. Each minimized complex was then analyzed by energy decomposition to derive electrostatic and van der Waals interaction terms for each receptor amino acid residue with any given nucleotide. All such energy terms were then reweighted via partial least squares fitting (performed with the Simca-P program [25]) with experimentally determined K_i values.

2.7. Miscellaneous

Protein was determined using the Bio-Rad DC protein assay. Data shown in Tables 1, 2 and 4 were obtained by non-linear regression analysis of GTPase- and AC competition experiments using the Prism 4.0 software (GraphPad, San Diego, CA, USA).

3. Results and discussion

3.1. Affinities of G_s- and G_i-proteins for NTPs, NTPγSs and NppNHps

Competition of agonist-stimulated steady-state [γ-^{32}P]GTP hydrolysis in receptor-G_α fusion proteins expressed in Sf9 insect cell membranes by unlabeled nucleotides constitutes a sensitive method to determine nucleotide-affinities of G-proteins [9,26]. The use of receptor-G_α fusion proteins was necessary to compare G_i and G_s directly since the GTPase assay with non-fused G_s is not sufficiently sensitive for a direct comparison with G_i [15,16]. As the affinity of non-fused and fused G_i for GTPγS is similar [16], we assume that the fusion does not grossly alter the nucleotide binding properties of G_α, rendering fusion proteins a valid system for the analysis of nucleotide-affinities. Both G_s and G_i bound NTPs in the order of affinity GTP > ITP > XTP > UTP > CTP (Table 1). Substitution of the γ-phosphate by γ-thiophosphate in GTP substantially increases nucleotide-affinity for G_α [1,2,11]. Our data with GTP/GTPγS and receptor-G_α fusion proteins are in accordance with the literature. We noted that for the low-affinity pyrimidine NTPs, UTP and CTP, introduction of the γ-thiophosphate increased nucleotide-affinity much more profoundly than for the higher-affinity purine NTPs. These data indicate that the unique interaction of the γ-thiophosphate with G_α [27] compensates, to a significant extent, for the suboptimal interaction of uracil and cytidine with the nucleotide-binding pocket [9]. In fact, the increase in affinity for UTPγS relative to UTP both at G_s and G_i was so profound that the affinity of this NTPγS approached that of the endogenous G-protein substrate GTP. Although GppNHp is, like GTPγS, hydrolysis-resistant, GppNHp binds to G-proteins with much lower affinity than GTPγS [1,2,11]. Again, our data are in agreement with the literature. The order of affinity of NppNHps for G_s and G_i was the similar as for NTPs, with the β,γ-imido group having either negative or only slightly positive influence on nucleotide-affinity of G-proteins. Collectively, our data show that G-proteins bind both purine and pyrimidine nucleotides, with a strong preference for guanine nucleotides. Introduction of a γ-thiophosphate group into NTPs generally increases nucleotide-affinity, and this effect is particularly striking for UTP and CTP.

Table 1 – Affinities of G_s- and G_i-proteins for NTPs, NTPγSs and NppNHps

Nucleotide	β_2AR-$G_{s\alpha L}$ (K_i, μM)	FPR-$G_{i\alpha 2}$ (K_i, μM)
GTP	0.18 (K_m)	0.11 (K_m)
ITP	10	3.8
XTP	74	27
UTP	910	1100
CTP	3700	4100
GTPγS	0.0058 (31)	0.0079 (14)
ITPγS	0.059 (170)	0.064 (59)
XTPγS	3.2 (23)	1.7 (16)
UTPγS	0.19 (4800)	0.70 (1600)
CTPγS	3.0 (1200)	3.1 (1300)
GppNHp	0.40 (0.45)	0.15 (0.73)
IppNHp	6.3 (1.6)	1.3 (2.9)
XppNHp	5700 (0.01)	310 (3.6)
UppNHp	4900 (0.76)	>10000 (<0.41)

Agonist-stimulated GTPase activity in Sf9 membranes expressing β_2AR-$G_{s\alpha L}$- and FPR-$G_{i\alpha 2}$ fusion proteins was determined as described in Section 2. Reaction mixtures contained 100 nM [γ-^{32}P]GTP (0.2 μCi/tube) and nucleotides between 1 nM and 10 mM as appropriate to obtain saturated concentration/response curves. With all nucleotides at sufficiently high concentrations, depending on their affinity, complete inhibition of enzyme activity was achieved. Data were analyzed by non-linear regression. Competition isotherms were best fitted to monophasic inhibition functions. For the substrate GTP, K_m values are provided. Data shown are the means of three independent experiments performed in duplicates. S.D. values were generally less than 10–15% of the means. In parentheses, the ratios of K_i values (NTP/NTPγS and NTP/NppNHp) are listed. The K_m values of agonist-stimulated GTP hydrolysis were taken from [8] and [16].

3.2. Inhibition of the catalytic activity of C1·C2·forskolin by NTPs, NTPγSs and NppNHps

In order to compare the affinities of nucleotides for G-proteins relative to AC, we conducted competition studies analogous to those with receptor-G_α fusion proteins using the purified catalytic subunits of AC, C1 and C2 (Table 2). Those studies were performed only in the presence of the direct AC activator, forskolin, but not in the presence of the allosteric AC activator, $G_{s\alpha}$ [14,28]. This experimental design was chosen to avoid any interference of nucleotide interaction with $G_{s\alpha}$ on AC activity measurements. Moreover, we conducted our AC studies both in the presence of Mg^{2+} and Mn^{2+} since the type of divalent cation present can have an impact on nucleotide-affinity [12,20,28] and since GTPase studies were all conducted in the presence of Mg^{2+}, allowing for direct data comparison.

Table 2 – Inhibition of the catalytic activity of C1·C2·forskolin by NTPs, NTPγSs and NppNHps

Nucleotide	C1·C2·forskolin·Mn^{2+} (K_i, μM)	C1·C2·forskolin·Mg^{2+} (K_i, μM)
ATP	620 (K_m)	560 (K_m)
GTP	1900	1100
ITP	1500	1900
XTP	3200	2400
UTP	2000	2400
CTP	2400	2200
ATPγS	170 (3.6)	1900 (0.30)
GTPγS	51 (37)	31 (35)
ITPγS	10 (150)	15 (130)
XTPγS	38 (84)	59 (41)
UTPγS	16 (125)	26 (92)
CTPγS	16 (150)	21 (100)
AppNHp	290 (2.1)	4000 (0.14)
GppNHp	780 (2.4)	2500 (0.44)
IppNHp	230 (6.5)	840 (2.3)
XppNHp	1600 (2.0)	2100 (1.1)
UppNHp	410 (5.9)	610 (3.9)

Catalytic activity of C1·C2 in the presence of 100 μM forskolin was determined with 40 μM ATP and 1 μCi [α-^{32}P]ATP as substrate with $MnCl_2$ (10 mM) or $MgCl_2$ (10 mM) and nucleotides between 1 μM and 10 mM as appropriate to obtain saturated concentration/response curves. With all nucleotides at sufficiently high concentrations, depending on their affinity, complete inhibition of enzyme activity was achieved. Data were analyzed by non-linear regression. Competition isotherms were best fitted to monophasic inhibition functions. For the substrate ATP, K_m values are provided. Data shown are the means of three independent experiments performed in duplicates. S.D. values were generally less than 10–15% of the means. In parentheses, the ratios of K_i values (NTP/NTPγS and NTP/NppNHp) are listed. The K_m values for C1·C2 were taken from [29].

Relative to the AC substrate ATP, other purine and pyrimidine nucleotides exhibited 3–6-fold lower affinities for C1·C2 in the presence of Mn^{2+}. However, the base-specificity of AC was much less pronounced than that of G-proteins. This was evident for NTPs, NTPγSs and NppNHps. In contrast to the GTP/GTPγS pair at G-proteins, there was only little increase in affinity for C1·C2 when comparing ATP and ATPγS in the presence of Mn^{2+}. For other NTPs, the exchange of the γ-phosphate against γ-thiophosphate increased nucleotide-affinity ~40–150-fold. However, compared to the effect of the γ-thiophosphate substitution in pyrimidine nucleotides on G-protein affinity, the impact of this substitution on C1·C2-affinity was moderate. The β,γ-imido substitution increased C1·C2-affinity of most nucleotides moderately, whereas for G-proteins, a decrease in affinity was predominant. The exchange of Mn^{2+} against Mg^{2+} substantially reduced the affinity of C1·C2 for ATPγS and AppNHp. These data are in contrast with the fact that the ATP-affinity of C1·C2 is similar in the presence of Mn^{2+} and Mg^{2+} [12,29]. These differences point to substantial differences in the molecular interactions of ATPγS and AppNHp on one hand and ATP on the other hand, with C1·C2. For the other nucleotides, exchange of Mn^{2+} against Mg^{2+} did not largely change affinities for C1·C2. Collectively, C1·C2 binds both purine and pyrimidine nucleotides with only moderate preference or no preference for adenine nucleotides relative to other purine and pyrimidine nucleotides. In addition, introduction of a γ-thiophosphate substantially increases the affinity of C1·C2 for non-adenine nucleotides.

3.3. *Molecular modeling of the interaction of C1·C2·forskolin with NTPs, NTPγSs and NppNHps*

Upon energetic decomposition and statistical reweighting, the computationally derived complexes between purine and pyrimidine nucleotides reported in Table 2 with the C1·C2 receptor produced pharmacophore models that correlated strongly with experimental data. In the case of ligands binding to C1·C2 in the presence of excess Mn^{2+}, the calculated K_i values (pK_i(calc)) and experimentally determined K_i values (pK_i(exp)) (plotted in Fig. 1a) exhibited a correlation of $R^2 = 0.93$, a leave-one-out cross-validation score of $Q^2 = 0.71$, and a root-mean-squared error (RMSE) of only 0.25. In the case of excess Mg^{2+} (Fig. 1b), the correlation was similar ($R^2 = 0.89$, $Q^2 = 0.75$, RMSE = 0.31). These correlations show that, given an approximate account for dynamic and entropic effects (as is accomplished in the reweighting of energy terms), it is possible to develop a consistent and predictive model for the interaction of NTPs, NTPγSs and NppNHps with C1·C2. Furthermore, given the manual docking strategy of aligning

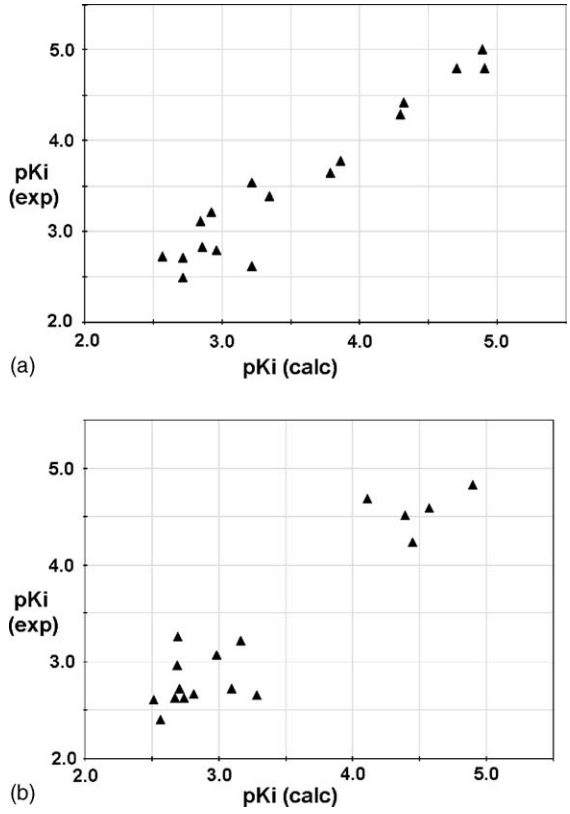

Fig. 1 – Correlation plots for calculated vs. experimental inhibition constants for NTPs, NTPγSs and NppNHps interacting with AC (C1·C2·forskolin). The experimentally determined K_i values shown in Table 2 were converted in pK_i values (pK_i(exp)). Calculated K_i values (pK_i(calc)) were derived as described in Section 2. (a) Data for an excess of Mn^{2+} and (b) data for an excess of Mg^{2+}.

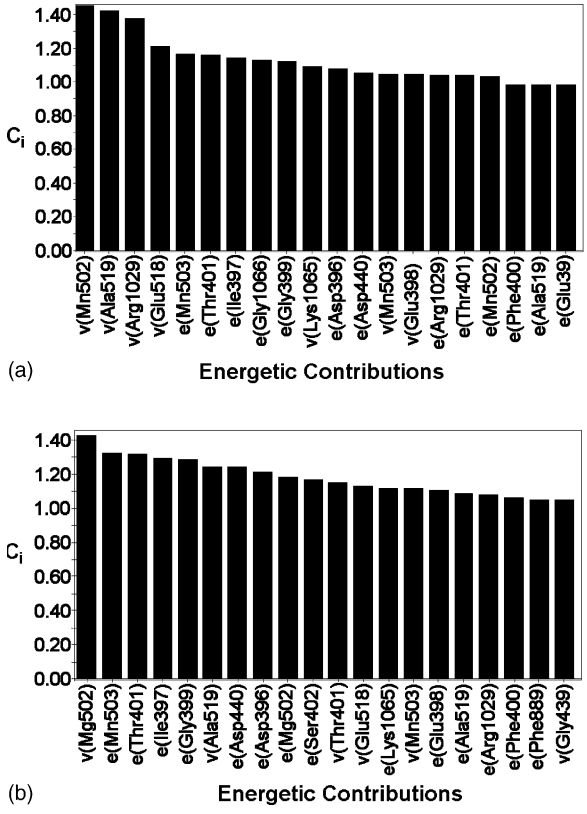

Fig. 2 – The 20 largest reweighted energy coefficients for the trained expressions relating calculated inhibition constants to experimental values for NTPs, NTPγSs and NppNHps interacting with AC (C1·C2·forskolin). Specific coefficients (C_i) relate the van der Waals interactions (prefixed with "v") and electrostatic interactions (prefixed with "e") between specific receptor amino acid residues and the manifold of ligands. (a) Data for an excess of Mn^{2+} and (b) data for an excess of Mg^{2+}.

the phosphate tail of nucleotides to the originally co-crystallized ATPαS, nucleotide ligands bind to C1·C2 in a consistent manner that is similar to that determined in the original crystal structure [21].

Information about the pharmacophore models for nucleotide binding to the Mn^{2+}-rich and Mg^{2+}-rich C1·C2 receptor is shown in Fig. 2a and b, respectively, in which the 20 largest reweighted coefficients of the following expression:

$$pK_i = \sum_i C_i^{ele} E_i^{ele} + \sum_j C_j^{vdW} E_j^{vdW}$$

are reported. In both cases, the receptor metal atoms have large coefficients for both their van der Waals and electrostatic terms. This is indicative for the relevance of metal atoms in the binding mechanisms that is reflected consistently across the set of 17 nucleotides examined herein. Both in the presence of Mn^{2+} and Mg^{2+}, the van der Waals coefficient for the metal binding of the α- and β-phosphate groups (assigned residue number 502) is the largest of all, a fact that agrees well with our observation that the nature of this metal (i.e. Mg versus Mn) has significant effects on the binding affinity of different ligands. The coefficients for the other metal (residue 503; Mn^{2+} in both cases) are smaller than those for Mg/Mn 502 but are still among the largest. For Mn 503, the coefficient for electrostatic interactions is larger than for van der Waals interactions, a fact that is not surprising since it is primarily responsible for binding the compact dianionic γ-phosphate tail. Among the other residues with coefficients appearing in the top 20 lists reported in Fig. 2, the majority of them (Asp 396, Ile 397, Glu 398, Gly 399, Thr 401, Ser 402, Asp 440, Glu 518, Ala 519, Arg 1029, Lys 1065, and Gly 1066) interact primarily with the phosphate tail. Phe 400 and Arg 1029 interact primarily with the ribosyl residue, and Gly 439 and Phe 889 bind the nucleotide bases. These data suggest that a key criterion for high ligand potency is a binding mode that permits optimal phosphate interactions with its corresponding pocket. The importance of the phosphate tail of nucleotides for interaction with C1·C2 could also explain the substantial dependence on phosphate tail character, with γ-thiophosphate-containing nucleotides being the most potent inhibitors. Under Mn^{2+} conditions, the NppNHp structure appears to be consistently superior to the normal triphosphates. However, matters become more complicated under Mg^{2+} conditions, where ATP and GTP are significantly favored relative to AppNHp and GppNHp, respectively. This appears to be due to the relative bulk of the base moiety in adenine and guanine nucleotides, preventing a relaxation of the ribosyl group that would otherwise lead (in the NppNHp cases) to more favorable H-bonding interactions with Ser 1028 and Arg 1029.

3.4. *Comparison of the affinities of NTPs, NTPγSs and NppNHps for G-proteins relative to C1·C2*

In the next step, we calculated the ratios of the K_i values of nucleotides for C1·C2 and G-proteins using Mg^{2+} conditions (Table 3). These calculations revealed that GTP, GTPγS and GppNHp exhibited several thousand-fold selectivity for G_s- and G_i-proteins relative to C1·C2. ITP and IppNHp also exhibited at least 190-fold selectivity for G-proteins relative to C1·C2. For XTP, XTPγS and UTPγS the selectivity for G-proteins decreased to 20–140-fold. UTP, CTPγS and XppNHp (G_i only) showed a G-protein preference of just 2–7-fold. CTP and UppNHp showed moderate preference for C1·C2 relative to G-proteins.

3.5. *Inhibition of holo-ACs by NTPs, NTPγSs and NppNHps*

While the studies with C1·C2 had the advantage of the absence of G-proteins and allowed direct correlation of experimental and molecular modeling data, nonetheless, the C1·C2 system is artificial in the sense that it lacks the transmembrane domains of AC [14,28]. Therefore, it was important to examine whether holo-ACs in cell membranes are inhibited by NTPs, NTPγSs and NppNHps as well. Since cell membranes endogenously express various G-proteins, it was essential for these studies to eliminate, as far as possible, the potential impact of G-proteins on AC activity. We studied two systems, i.e. the $G_{s\alpha}$-deficient S49 *cyc*$^-$ lymphoma cell membranes and membranes from uninfected Sf9 membranes (Table 4). The impact of G_i-proteins on AC activity in S49 *cyc*$^-$ membranes was eliminated by conducting AC assays in the presence of a high (10 mM) concentration of Mn^{2+} [20].

Table 3 – Comparison of the affinities of NTPs, NTPγSs and NppNHps for G-proteins relative to purified catalytic AC subunits and holo-AC

Nucleotide	K_i C1·C2/K_i β_2AR-$G_{s\alpha L}$	K_i S49 *cyc*$^-$ AC/K_i β_2AR-$G_{s\alpha L}$	K_i C1·C2/K_i FPR-$G_{i\alpha 2}$	K_i S49 *cyc*$^-$ AC/K_i FPR-$G_{i\alpha 2}$
GTP	6100	3700	10000	6100
ITP	190	60	500	160
XTP	32	7.4	89	20
UTP	2.6	1.2	2.2	1.0
CTP	0.60	0.27	0.53	0.24
GTPγS	5300	71000	3900	52000
ITPγS	250	350	210	300
XTPγS	18	6.3	35	12
UTPγS	140	79	37	21
CTPγS	7.0	24	6.8	23
GppNHp	6300	7500	17000	20000
IppNHp	130	56	650	270
XppNHp	0.37	>3.6	6.8	>32
UppNHp	0.12	0.51	<0.06	<0.25

The K_i values for NTPs, NTPγSs and NppNHps shown in Tables 1, 2 and 4 were used as basis for calculations of K_i value ratios. Note that C1·C2 was analyzed in the presence of Mg^{2+}, while the S49 *cyc*$^-$ lymphoma cell membrane were studied in the presence of Mn^{2+}. GTPase assays were conducted in the presence of Mg^{2+}. In case of GTP, K_m values of the GTPase activities of $G_{s\alpha}$ and $G_{i\alpha}$ were used for calculations.

In S49 *cyc*$^-$ membranes, AC (reflecting AC isoforms 6 and 7) [30] exhibited 4–7-fold selectivity for ATP relative to the other purine and pyrimidine nucleotides. Introduction of the γ-thiophosphate group substantially increased the affinities of NTPγSs with the exception of GTPγS. In contrast, with the exception of IppNHp, introduction of the β,γ-imido group reduced nucleotide-affinity for AC.

Sf9 membranes express a $G_{s\alpha}$-like G-protein that exerts stimulatory effects on AC [15]. Similar to mammalian $G_{s\alpha}$-proteins, GTPγS activates the insect cell $G_{s\alpha}$-like G-protein with high affinity [15]. We activated the $G_{s\alpha}$-like G-protein with AlF_4^- that forms a complex with GDP [27]. Since GTPγS and GDP-AlF_4^- are similarly efficient G-protein activators, we would have expected GTPγS to be without effect on AC activity stimulated by GDP-AlF_4^- if GTPγS had bound only to the G-protein. However, GTPγS and the other nucleotides inhibited Sf9 insect cell AC with K_i values in the high micromolar to millimolar range, pointing to a direct interaction of nucleotides with AC. Similar to the observations made for the S49 *cyc*$^-$ membranes, insect cell AC showed a moderate preference for ATP relative to the other purine nucleotides (up to 10-fold) and somewhat greater selectivity (up to 30-fold) for pyrimidine nucleotides. The AC inhibition patterns observed for NTPγSs and NppNHps in Sf9 membranes were very similar to the patterns observed in S49 *cyc*$^-$ membranes. These data show that the interactions of purine and pyrimidine nucleotides with distantly related membranous holo-ACs are quite similar.

We noted that the holo-ACs discriminated between ATP and non-cognate NTPs much better than C1·C2. Moreover, NTPγSs inhibited the catalytic activity of C1·C2 rather non-selectively, whereas holo-ACs showed a clear preference for hypoxanthine, xanthine, uracil and cytosine relative to guanine. The preference for inhibition by IppNHp relative to inhibition by GppNHp was also more pronounced for holo-ACs than for C1·C2.

Table 4 – Inhibition of AC in S49 *cyc*$^-$ lymphoma and Sf9 insect cell membranes by NTPs, NTPγSs and NppNHps in the presence of Mn^{2+}

Nucleotide	S49 *cyc*$^-$ (K_i, μM)	Sf9 (K_i, μM)
ATP	150 (K_m)	100 (K_m)
GTP	670	440
ITP	600	990
XTP	550	950
UTP	1100	2200
CTP	1000	2900
GTPγS	410 (1.6)	400 (1.1)
ITPγS	21 (29)	20 (50)
XTPγS	20 (28)	25 (38)
UTPγS	15 (73)	51 (43)
CTPγS	71 (14)	33 (88)
GppNHp	3000 (0.22)	2400 (0.18)
IppNHp	350 (1.7)	480 (2.1)
XppNHp	>10000 (<0.06)	>10000 (<0.10)
UppNHp	2500 (0.44)	2800 (0.80)

AC activity in S49 *cyc*$^-$ lymphoma and Sf9 insect cell membranes was determined with 40 μM ATP and 1 μCi [α-^{32}P]ATP as substrate in the presence of 100 μM forskolin and 10 mM $MnCl_2$ and nucleotides between 1 μM and 10 mM as appropriate to obtain saturated concentration/response curves. With all nucleotides at sufficiently high concentrations, depending on their affinity, complete inhibition of enzyme activity was achieved. In case of Sf9 membranes, reaction mixtures also contained 10 mM NaF plus 10 μM $AlCl_3$ to maximally activate AC. Data were analyzed by non-linear regression. Competition isotherms were best fitted to monophasic inhibition functions. For the substrate ATP, K_m values are provided. Data shown are the means of three independent experiments performed in duplicates. S.D. values were generally less than 10–15% of the means. In parentheses, the ratios of K_i values (NTP/NTPγS and NTP/NppNHp) are listed.

3.6. *Comparison of the affinities of NTPs, NTPγSs and NppNHps for G-proteins relative to holo-AC in S49 cyc*$^-$ *lymphoma cell membranes*

In view of the fact that the inhibition profiles for holo-ACs differed somewhat from the inhibition profiles of C1·C2

(compare Tables 2 and 4), we calculated the ratios of the K_i values of nucleotides for AC in S49 *cyc*$^-$ membranes and G-proteins (Table 3). However, despite the differences between the two ACs, the overall pattern for the holo-AC/G-protein K_i value ratios was quite similar to the pattern observed for the C1·C2/G-protein K_i value ratio. Since the S49 *cyc*$^-$ lymphoma cell membrane AC discriminated very well between GTPγS and the other NTPγSs, the selectivity ratio of GTPγS for G-proteins relative to AC surpassed 50,000. The selectivity ratios of GTP and GppNHp for G-proteins relative to S49 *cyc*$^-$ lymphoma cell AC were also high (~4000–20,000-fold), but they decreased dramatically for all other nucleotides and were particularly low for UTP, CTP and UppNHp.

4. Conclusions

Our present study shows that both G-proteins and ACs bind purine and pyrimidine nucleotides. Thus, neither are G-proteins completely specific for guanine nucleotides nor is AC specific for adenine nucleotides. Another similarity between the two classes of signal transduction proteins is that γ-thiophosphate derivatives exhibit higher affinities for their target proteins than NTPs. However, despite these similarities, there are also striking differences in the interactions of G-proteins and AC with nucleotides. Specifically, G-proteins are highly selective for guanine nucleotides relative to other purine and pyrimidine nucleotides. This justifies why the initial term N-protein (for nucleotide-binding protein) was later replaced by the term G-protein (for guanine nucleotide-binding protein [1,2]. Similar rank orders of affinities of nucleotides as for the receptor-G_α fusion proteins reported in this paper are expected for purified G-proteins, but the absolute affinities of purified G-proteins for nucleotides are probably lower [1,2,9]. In contrast AC, most notably the purified catalytic subunits C1·C2, exhibit rather promiscuous interactions with purine and pyrimidine nucleotides. These data support the concept that the nucleotide-binding site of AC is conformationally much less constrained than the nucleotide-binding site of G-proteins [9,12,20,26,29,31]. Our molecular modeling studies confirm this concept. The different conformational constraints in G-proteins and AC are also reflected by the fact that guanine nucleotides bind to G-proteins with nanomolar to submicromolar affinities, whereas adenine nucleotides bind to AC only with submillimolar affinities.

The result of the differential interaction of nucleotides with G-proteins and AC is that guanine nucleotides exhibit an excellent (up to 71,000-fold) selectivity for G-proteins relative to AC. In contrast, the G-protein selectivity of other purine and pyrimidine nucleotides is much less pronounced (<1000-fold). Although a G_s-protein selectivity of 350 for ITPγS relative to the S49 *cyc*$^-$ lymphoma AC seems to be excellent on first glance, this apparently large selectivity, nonetheless, has profound experimental consequences as is evidenced by the biphasic (firstly G-protein-stimulatory and secondly directly AC-inhibitory) effects of this nucleotide in S49 wild-type and S49 *cyc*$^-$ lymphoma cell membranes [12]. Even more pronounced biphasic effects on AC activity in S49 wild-type lymphoma cell membranes were observed for UTPγS that exhibits a lower (80–140-fold) G_s-protein selectivity than ITPγS (250–350-fold) [12]. Based on the properties of these two NTPγSs, it is conceivable that dual effects of nucleotides on G-proteins and AC become more relevant with reduced G-protein-selectivity. Thus, direct AC inhibition is the most likely reason for the hitherto unexplained inhibitory effects of NTPs at millimolar concentrations on AC activity reported over a period of more than 30 years [6,10,32,33]. It is also possible that the apparently low efficacy of some nucleotides at supporting G_s-mediated AC activation, specifically UTPγS, CTP and CTPγS [5,6,9,12], is at least partially due to dual G_s-protein activation and AC inhibition, preventing those nucleotides from exhibiting their true G_s-protein-stimulatory potential. Thus, caution must be exerted when using non-guanine nucleotides as tools to probe distinct active G-protein states with AC activity as read-out. An important aspect in these studies is to additionally assess AC-independent parameters such as high-affinity agonist binding (disruption of ternary complex formation by nucleotides) and NTP hydrolysis [7–9,34] in order to prevent bias in data interpretation arising from dual interaction of nucleotides with G-proteins and AC.

What may be the physiological relevance of our data? Given the high preference of G-proteins for guanine nucleotides relative to other purine nucleotides and pyrimidine nucleotides, it is likely that under most physiological conditions, G-protein activation by guanine nucleotides dominates. However, with respect to the interaction of ACs with non-cognate nucleotides the situation is more complicated. Since the base-specificity of AC is much less pronounced than that of G-proteins and intracellular UTP- and CTP concentrations can reach concentrations in the 1–10 mM range [35], it is well possible that these nucleotides act as endogenous AC inhibitors in vivo. In order to substantiate this hypothesis, studies correlating cellular cAMP formation with intracellular UTP- and CTP concentrations will be necessary. Finally, the finding that ACs do not only bind purine nucleotides but also pyrimidine nucleotides with similar affinity may open novel avenues for the design of potent and selective AC inhibitors. The design of novel pyrimidine nucleotide-based AC inhibitors should be greatly facilitated by the validated computer model presented in this study.

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