DRUG DEVELOPMENT RESEARCH 42:98–108 (1997)

Research Article

Riboflavin: Inhibitory Effects on Receptors, G-Proteins, and Adenylate Cyclase

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Strategy, Management and Health Policy				
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ABSTRACT Riboflavin inhibited binding of both agonist and antagonist radioligands to rat brain A_1-adenosine receptors with K_i values of approximately 10 μM. In an adenylate cyclase assay with membrane preparations from either rat adipocytes or DDT MF-2 cells, both of which contain A_1-adenosine receptors, riboflavin inhibited isoproterenol-stimulated cyclase activity with an IC_{50} of approximately 20 μM. However, the inhibition of cyclase by riboflavin was not reversed by an A_1-selective antagonist, nor by pretreatment with pertussis toxin. Thus, neither A_1-receptors nor G_i-proteins appear critically involved in the inhibition of cyclase by riboflavin. Riboflavin did block the stimulation by an adenosine analog of [^{35}S]GTPγS binding in rat cerebral cortical membranes. However, riboflavin also inhibited the stimulation by fMLP of [^{35}S]GTPγS binding in HL-60 cell membranes. Riboflavin inhibited forskolin-stimulated cyclase in membranes from DDT MF-2 cells > rat adipocytes > PC12 cells, hamster CHO M2 cells, and wild-type S49 cells. There was virtually no inhibition of forskolin-stimulated cyclase in membranes of human platelets, rat cerebral cortex, or cyc$^-$S49 cells lacking G_s-proteins. The calcium-stimulated cyclase in rat cerebral cortical membranes was inhibited by riboflavin. A preincubation of membranes with riboflavin markedly enhanced the inhibition for DDT MF-2 and wild-type and cyc$^-$S49 membranes. The extent of inhibition in the different cell lines was dependent on the agent used to stimulate cyclase. Riboflavin, like the P-site inhibitor 2´,5´-dideoxyadenosine, was more potent and efficacious when manganese instead of forskolin was used as the stimulant. However, unlike the P-site inhibitor, riboflavin did not markedly inhibit GppNHp- or fluoride-stimulated cyclase. Riboflavin at low micromolar concentrations appears to have three possibly interrelated effects on second messenger systems subserved by G-proteins. These are antagonism at A_1-adenosine receptors, inhibition of turnover of guanyl nucleotides at G-proteins, and inhibition of adenylate cyclase. Drug Dev. Res. 42:98–108, 1997.

Key words: adenylate cyclase; P-site; adenosine analogs; xanthines; forskolin; G-proteins; A_1-adenosine receptors

INTRODUCTION

Riboflavin (vitamin B_2) serves as a precursor of riboflavin monophosphate (FMN)* and flavin-adenine dinucleotide (FAD) [Cooperman and Lopez, 1991]. Other possible functions or possible sites of action have not previously been defined. Pharmacologically, riboflavin can protect against ischemic damage [Hultquist et al., 1993; Betz et al., 1994] and has been stated to inhibit guanylate cyclase [Galagan et al., 1991], presumably through effects of dihydroriboflavin on oxidation states

*Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FAD, flavin-adenine dinucleotide; fMLP, N-formyl-Met-Leu-Phen; Guanosine 5´-0-(3-thiotriphosphate), GTPγS; 5´-Guanylateimidodiphosphate, GppNHp; 5´N-ethylcarboxamidoadenosine, NECA; FMN, riboflavin 5´-phosphate; R-PIA, N^6-R-phenylisopropyladenosine.

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Received 20 November 1997; Accepted 1 December 1997

of heme proteins and guanylate cyclase, respectively. We now report that riboflavin has micromolar affinity presumably as an antagonist for A_1-adenosine receptors. In addition, riboflavin caused inhibition of adenylate cyclase that was both A_1-receptor- and G_i-protein-independent, and inhibition of turnover of guanyl nucleotides at G-proteins.

MATERIALS AND METHODS

Materials

Riboflavin, 2´,5´-dideoxyadenosine, N^6-R-phenylisopropyladenosine (R-PIA), forskolin, 2-chloroadenosine, isoproterenol, and 8-cyclopentyl-1,3-dipropylxanthine were from Research Biochemicals International (Natick, MA). Prostaglandin E_1, GTPγS, GppNHp, adenosine deaminase, N-formyl-Met-Leu-Phe (fMLP), FMN, FAD, and pertussis toxin were from Sigma (St. Louis, MO). [^{3}H]Cyclohexyladenosine (sp. act. 30 Ci/mmol), [^{3}H]8-cyclopentyl-1,3-dipropylxanthine (sp. act. 120 Ci/mmol), [^{32}P]cyclic AMP (sp. act. 800 Ci/mmol), and [^{3}H]cyclic AMP (sp. act. 34 Ci/mmol) were from NEN Life Sciences (Boston, MA). [^{35}S]GTPγS (sp. act. 1,275 Ci/mmol) was from Amersham (Chicago, IL).

Cell lines were from the following sources: Rat pheochromocytoma PC12 cells were from Dr. G. Guroff (NIH, Bethesda, MD), hamster leiomyosarcoma DDT MF-2, human leukemic HL-60 cells, mouse wild-type, and cyc$^-$S49 cells from the American Type Culture Collection (Rockville, MD) and chinese hamster ovary CHO M2 cells transfected with M_2 muscarinic receptors from Dr. J. Wess (NIH). Rat adipocyte membranes were provided by Dr. C. Londos (NIH). Platelets were from the Transfusion Medicine Branch (NIH).

Cell Culture

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with a 6% fetal calf serum, 6% horse serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. DDT MF-2 and CHO M2 cells were grown in DMEM with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. S49 cells were grown in DMEM with 10% horse serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. HL-60 cells were grown in suspension in Roswell Park Memorial Institute 1640 medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin and were differentiated prior to use in medium containing dimethylsulfoxide for 48 h. Cells were kept at 37°C in an atmosphere enriched in CO_2.

Membrane Preparation for Binding Assays

Frozen brains from 25 rats (Pel-Freez Biological; Rogers, AR) were placed in ice-cold 0.32 M sucrose to thaw. Cerebral cortices were removed and placed in centrifuge tubes, each with 15 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4). Tissues were homogenized with a polytron (setting #6) for 10 sec and ice-cold 50 mM Tris-HCl buffer (pH 7.4) was added to give a volume of 35 ml, followed by centrifugation at 1,000*g* for 15 min at 4°C. The supernatant was removed and recentrifuged at 35,000*g* for 15 min at 4°C. The pellets from the second centrifugation were resuspended in the ice-cold 50 mM Tris-HCl buffer (pH 7.4), homogenized with the polytron (setting #6) for 10 sec and centrifuged at 35,000*g* for 15 min at 4°C. The final membrane pellets were stored at –70°C. Membranes were resuspended and diluted to a protein concentration of 2–3 mg/ml for the binding assay. Protein concentrations were determined by the BCA protein assay reagents (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard.

Radioligand Binding

Binding of [^{3}H]N^6-cyclohexyladenosine and [^{3}H]8-cyclopentyl-1,3-dipropylxanthine to A_1 adenosine receptors in rat cerebral cortical membranes was determined essentially as described [Bruns et al., 1980, 1987]. The [^{3}H]N^6-cyclohexyladenosine was incubated in 50 mM Tris-HCl (pH 7.4) containing 100 µl membrane suspension with 1.5 units/ml adenosine deaminase for 90 min at 25°C in a final volume of 0.5 ml. Competitive inhibition experiments with riboflavin were with 1 nM [^{3}H]N^6-cyclohexyladenosine. Saturation analyses were with 0.5–12 nM [^{3}H]N^6-cyclohexyladenosine and different concentrations of riboflavin. Nonspecific binding was defined in the presence of 10 µM 2-chloroadenosine. The [^{3}H]8-cyclopentyl-1,3-dipropylxanthine was incubated in 50 mM Tris-HCl (pH 7.4), containing 100 µl membrane suspension with 1.5 units/ml adenosine deaminase for 90 min at 25°C in a final volume of 0.5 ml. Competitive inhibition experiments with riboflavin were with 0.4 nM [^{3}H]8-cyclopentyl-1,3-dipropylxanthine. Saturation analyses were with 0.1–3.2 nM [^{3}H]8-cyclopentyl-1,3-dipropylxanthine and different concentrations of riboflavin. Nonspecific binding was defined in the presence of 10 µM 2-chloroadenosine. Binding reactions were terminated by filtration through Whatman GF/B filter, using a Brandel cell harvester (Brandel, Gaithersburg, MD). Filters were washed twice with 5 ml ice-cold buffer and placed in vials with 5 ml of Hydrofluor scintillation fluid, followed by counting for tritium.

Binding of [^{35}S]GTPγS to rat cerebral cortical membranes was determined as described [Lorenzen et al., 1993]. Briefly, rat cerebral cortical membranes were suspended in a buffer containing 50 mM Tris-HCl (pH 7.4),

3 units/ml adenosine deaminase, 100 mM NaCl, and 10 mM $MgCl_2$ at a protein concentration of 1–10 μg per tube. The membrane suspension was preincubated with 0.5 μM GDP and varying concentrations of R-PIA and/or riboflavin in a final volume of 450 μl buffer at 30°C for 20 min and then transferred to ice for 20 min. The [^{35}S]GTPγS was added in 50 μl buffer to a final concentration of 0.1 nM followed by incubation for 30 min at 30°C. Nonspecific binding was determined in the presence of 10 μM GTPγS. Incubations were terminated by filtration over GF/B glass fibers using a Brandel cell harvester, followed by washing with cold buffer and determination of radioactivity. Binding of [^{35}S]GTPγS to HL-60 membranes was conducted in a similar manner as described [Gierschik et al., 1991], using fMLP as the agonist to stimulate [^{35}S]GTPγS binding.

Membrane Preparation for Adenylate Cyclase Assays

The CHO M2, DDT MF-2, and S49 cells were removed from each culture flask by treatment for about 3 min with 0.5% trypsin in 0.53 mM EDTA, followed by centrifugation at 800*g* for 5 min. The pellet was washed twice with Krebs phosphate buffer (pH 7.4) containing 128 mM NaCl, 1.4 mM $MgCl_2$, 5.2 mM KCl, 10 mM Na_2HPO_4, and then once with ice-cold 50 mM Tris-HCl (pH 7.4). The cells were then homogenized using a Dounce homogenizer (15 strokes) and centrifuged at 35,000*g* for 15 min. The pellet was resuspended in ice-cold media containing 75 mM Tris-HCl (pH 8.12), 200 mM NaCl, 12.5 mM $MgCl_2$, and 1 mM dithiothreitol and frozen in 1 ml aliquots for subsequent use in adenylate cyclase assays. The PC12 cell membranes, platelet membranes, and brain membranes were prepared as described [Ukena et al., 1986]. Isolated fat cell plasma membranes were provided by Dr. C. Londos (NIH).

Adenylate Cyclase Assay

Assay of activity of membrane adenylate cyclase was determined essentially as described [Ukena et al., 1986]. Briefly, assays were conducted in a total volume of 250 μl of 50 mM Tris-HCl (pH 7.4), containing 5 mM $MgCl_2$, 10 μM GTP (1 μM for DDT MF-2 cells, 20 μM for fat cells), 20 μM EGTA, 1 unit adenosine deaminase, 5 mM creatine phosphate, 0.3 mg creatinine kinase, and 30 μg bovine serum albumin. Stimulants (Mn^{++}, forskolin, isoproterenol, 5´N-ethylcarboxamidoadenosine, prostaglandin E_1, fluoride, GppNHp, or calcium ions) and inhibitors (R-PIA, riboflavin, or 2´,5´-dideoxyadenosine) of adenylate cyclase were added, followed by a preincubation for 10 min at 37°C. Membranes and [α-^{32}P]ATP (0.9 μCi/tube) and ATP to a final concentration of 100 μM (20 μM for adipocytes) were then added, followed by incubation for 10 min at 37°C. Reactions were stopped by addition of 0.5 ml of trichloroacetic acid containing 0.25 ml of 1 mM cyclic AMP and 25 nCi [^{3}H]cyclic AMP. The cyclic AMP was isolated by a two-step chromatographic procedure, using Dowex 50 and alumina columns [Ukena et al., 1986]. Loss of [^{32}P]cyclic AMP during isolation was corrected by comparison with the loss of [^{3}H]cyclic AMP.

Analysis of Data

K_d and B_{max} values were determined by linear fitting of Scatchard plots. IC_{50} values were analyzed by nonlinear regression using the computer program GraphPad InPlot (Version 4.0, San Diego, CA). K_i values were calculated using the Cheng-Prusoff relationship. The statistical significance was assessed with Student's t-test using the computer program SYSTAT (Version 5.0, Evanston, IL).

RESULTS

The K_i value of riboflavin for inhibition of binding of the agonist [^{3}H]N^6-cyclohexyladenosine to A_1-adenosine receptors in rat cerebral cortical membranes was 6.6 ± 0.4 μM, while the K_i value for inhibition of binding of the antagonist [^{3}H]8-cyclopentyl-1,3-dipropylxanthine was 12.9 ± 1.6 μM (Fig. 1). A K_i value for riboflavin vs. binding of [^{3}H]R-PIA to rat A_1-adenosine receptors of 13 μM has been reported [Siddiqi et al., 1996]. Satura-

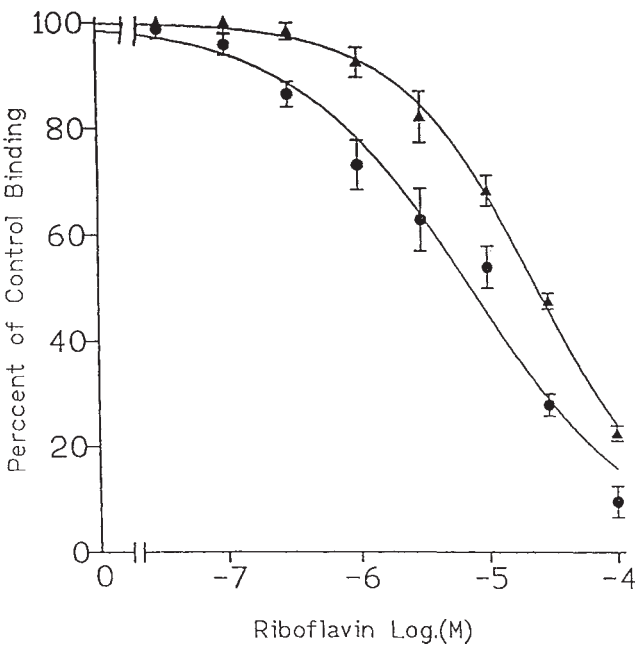


Fig. 1. Inhibition by riboflavin of binding of radioligands to A_1-adenosine receptors in rat cerebral cortical membranes. Values are means ± SEM (n = 3) for inhibition of binding of the agonist [^{3}H]N^6-cyclohexyladenosine (●) or the antagonist [^{3}H]8-cyclopentyl-1,3-dipropylxanthine (▲) by riboflavin, as described in Methods.

TABLE 1. Effect of Riboflavin on Bindng of an Agonist and an Antagonist, [^{3}H]8-Cyclopentyl-1,3-dipropylxanthine, to A_1-Adenosine Receptors in Rat Cerebral Cortical Membranes[a]

	Agonist [^{3}H]N^6-cyclohexyladenosine		Antagonist [^{3}H]8-cyclopentyl-1,3-dipropylxanthine	
Agent (μM)	K_d nM	B_{max} fmol/mg protein	K_d nM	B_{max} fmol/mg protein
Control	1.14 ± 0.08	414 ± 16	0.48 ± 0.08	891 ± 12
Riboflavin (3)	1.34 ± 0.24	326 ± 28**	0.52 ± 0.11	885 ± 21
Riboflavin (10)	2.30 ± 0.18**	325 ± 25**	0.56 ± 0.10	821 ± 11**
Riboflavin (30)	3.38 ± 0.56**	266 ± 20*	0.79 ± 0.05**	781 ± 13*

[a]Results are from saturation analysis of data presented in Figure 2. *$P < 0.01$, **$P < 0.05$.

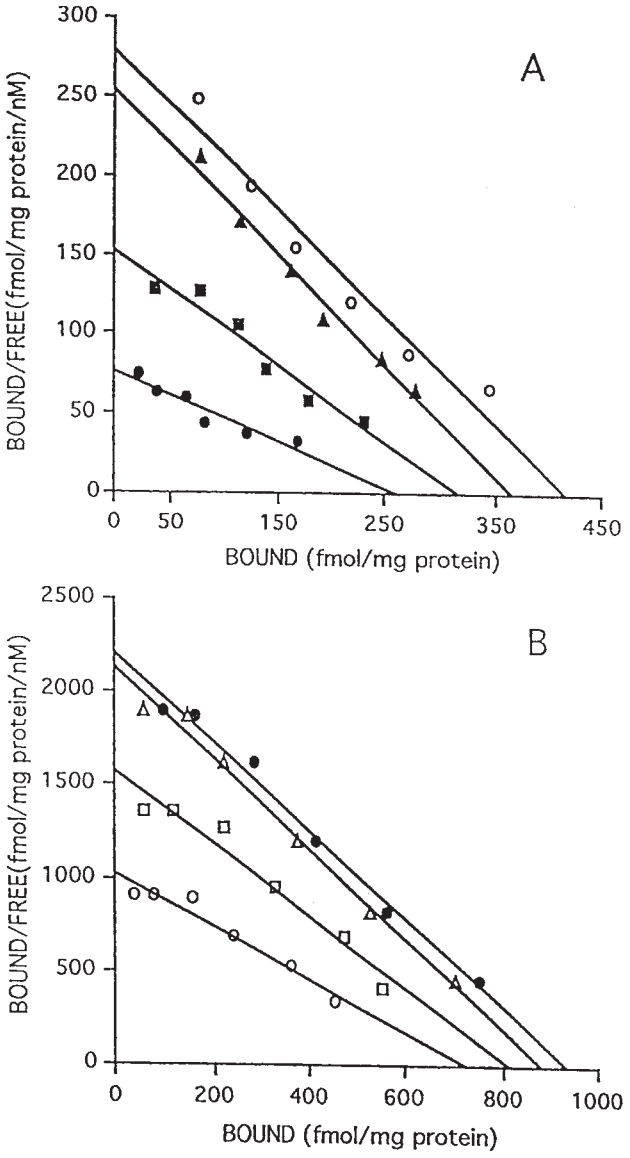


Fig. 2. Saturation analysis of effects of riboflavin on binding of radioligands to A_1-adenosine receptors in rat cerebral cortical membranes. (**A**) [^{3}H]N^6-cyclohexyl-adenosine: control ○; riboflavin 3 μM ▲; 10 μM ■; 30 μM ●. (**B**) [^{3}H]8-cyclopentyl-1,3-dipropylxanthine: control ●; riboflavin 3 μM △; 10 μM □; 30 μM ○ (see Methods).

tion analysis of the effect of 30 μM riboflavin on binding revealed that the K_d values for both ligands were increased, consistent with competitive inhibition. However, the B_{max} values for both ligands were decreased, an effect suggestive of some involvement of uncompetitive inhibition (Table 1, Fig. 2A,B). The effects of 100 μM riboflavin on binding were reversible on washing (data not shown). The presence of 10 μM GTP in the binding assay with [^{3}H]8-cyclopentyl-1,3-dipropylxanthine had no significant effect on the K_i value for riboflavin, consonant with riboflavin acting as an antagonist rather than an agonist (data not shown).

The effects of riboflavin on A_1-receptor-mediated stimulation of [^{35}S]GTPγS binding to G_i-proteins in membranes of rat cerebral cortex and on A_1-receptor-mediated inhibition of adenylate cyclase in membranes from DDT MF-2 cells and rat adipocytes were then investigated.

Riboflavin had nearly no effect alone on the binding of [^{35}S]GTPγS to rat cerebral cortical membranes, but inhibited the stimulatory effect of the A_1-adenosine receptor agonist R-PIA with an IC_{50} of about 20 μM (Fig. 3A). In membranes from differentiated HL-60 cells, riboflavin inhibited binding of [^{35}S]GTPγS both under basal conditions and when stimulated by fMLP (Fig. 3B).

Both R-PIA and riboflavin caused inhibition of isoproterenol-stimulated adenylate cyclase in rat adipocyte membranes, which maximized at about 50% (Fig. 4A). The inhibition by R-PIA was completely blocked by an A_1-adenosine receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine, while the inhibition by riboflavin was unaffected. Similar results were obtained with DDT MF-2 cell membranes (Fig. 4B). A prior treatment of DDT MF-2 cells with pertussis toxin to block G_i-protein-mediated inhibition of cyclase abolished the effect of R-PIA as expected, while having no effect on the inhibition by riboflavin (Fig. 5). Thus, riboflavin was not inhibiting adenylate cyclase through the A_1-adenosine receptor–G_i protein pathway. The extent of inhibition of adenylate cyclase in DDT MF-2 cell membranes was increased by preincubation of membranes with riboflavin for 5–20

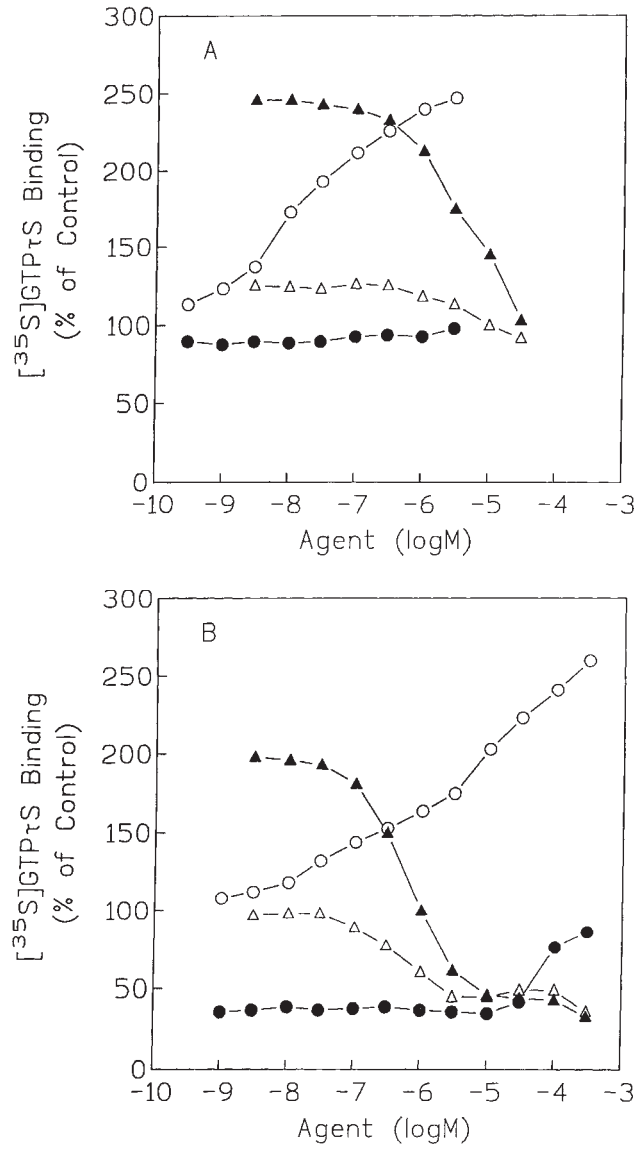


Fig. 3. Effect of riboflavin on [^{35}S]GTPγS binding. (**A**) Rat cerebral cortical membranes. (**B**) HL-60 membranes. Membranes were incubated with increasing concentrations of (**A**) R-PIA or (**B**) fMLP in the absence (○) or presence of 10 μM riboflavin (●), or with increasing concentrations of riboflavin in the absence (△) or presence (▲) of (**A**) 10 μM R-PIA and (**B**) 10 μM fMLP. Values are means (n = 3). Percent stimulation over control levels of binding of [^{35}S]GTPγS were (**A**) 208 ± 28% (n = 6) and (**B**) 204 ± 24% (n = 7). See Methods for assay.

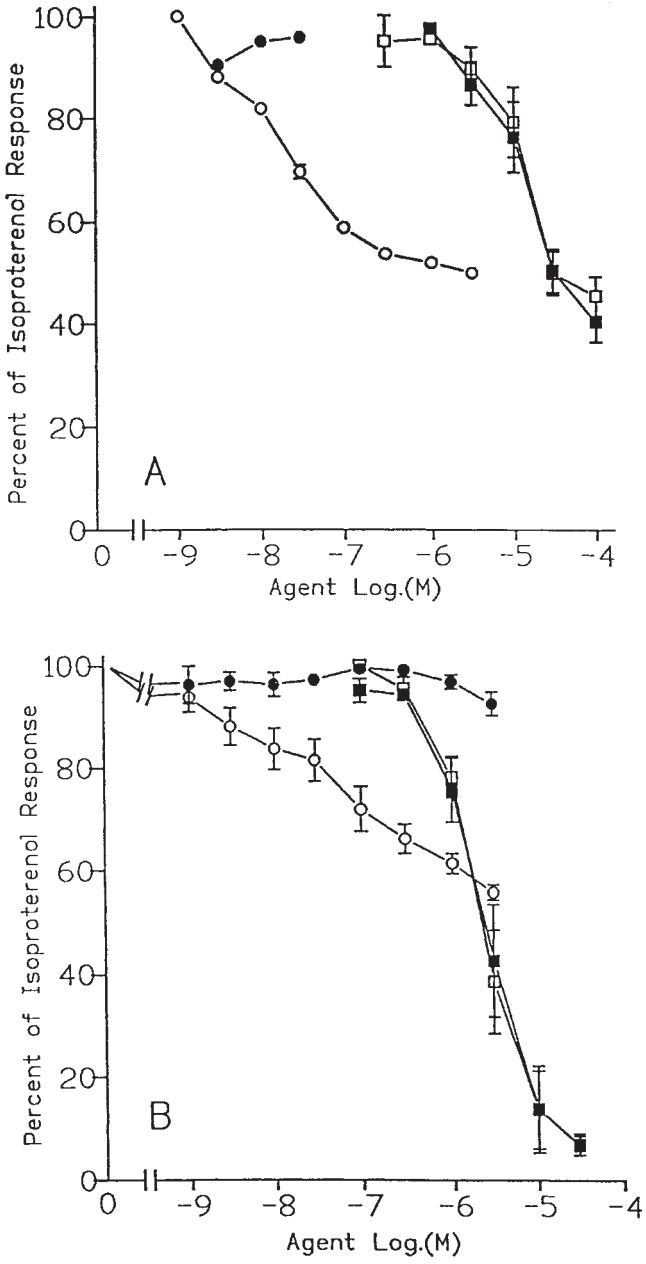


Fig. 4. Inhibition of adenylate cyclase activity by riboflavin and an adenosine analog: Lack of antagonism of riboflavin by an adenosine receptor antagonist. (**A**) Rat adipocyte membranes and (**B**) DDT MF-2 cell membranes. Inhibition of isoproterenol (10 μM)-stimulated cyclase by R-PIA (circles) or riboflavin (squares). Open symbols are in the absence and closed symbols in the presence of the A_1-adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (10 μM). See Methods for assay. Values are means ± SEM (n = 3). Error bars are in some cases smaller than the symbol.

min prior to addition of [^{32}P]ATP and assay (Table 2). FMN and FAD at 100 μM had no effect on cyclase activity in DDT MF-2 cell membranes (data not shown).

The inhibitory effects of riboflavin on adenylate cyclase were now compared to the effects of the P-site inhibitor 2′,5′-dideoxyadenosine. In DDT MF-2 cell membranes, both riboflavin and 2′,5′-dideoxyadenosine were more potent and efficacious as inhibitors against Mn^{2+}-stimulated cyclase than against forskolin-stimulated cyclase (Fig. 6A). In contrast, the adenosine analog R-PIA was more efficacious vs. forskolin-stimulated cyclase. 2′,5′-Dideoxyadenosine caused a marked inhibition of fluoride- and GppNHp-stimulated cyclase, while riboflavin did not (Fig. 6B). Altering the ATP concentration from 30 to 300

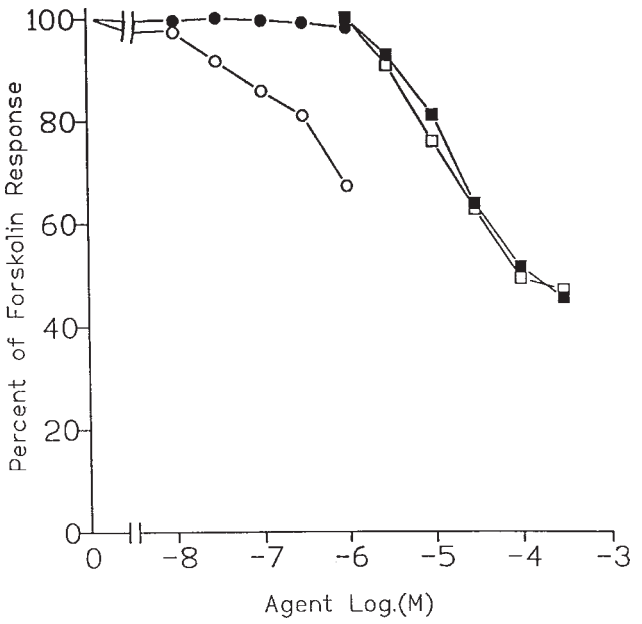


Fig. 5. Effect of pertussis toxin on the inhibition of adenylate cyclase by riboflavin and by an adenosine analog in DDT MF-2 cell membranes. Inhibitions are for R-PIA in control (○) and pertussis toxin-treated cells (●) and for riboflavin in control (□) and pertussis toxin-treated cells (■). Values represent average inhibition of the forskolin-stimulated adenylate cyclase of three determinations for each point from one experiment in which DDT MF-2 cells were incubated with pertussis toxin (200 ng/ml) for 16 h prior to preparation of membranes and assay. Similar results were obtained in a second experiment. See Methods for assay.

μM had no effect in DDT MF-2 cell membranes on the inhibition by riboflavin (data not shown).

In PC12 cell membranes, in which A_1-adenosine receptors are absent, both riboflavin and 2′,5′-dideoxyadenosine caused a marked inhibition of adenylate cyclase and both were more efficacious vs. Mn^{2+}-stimulated cyclase than vs. forskolin-stimulated cyclase (Fig. 7A). Riboflavin caused only a slight inhibition of N-ethylcarboxamidoadenosine-, fluoride-, and GppNHp-stimulated cyclase, while 2′,5′-dideoxyadenosine was quite effective (Fig. 7B).

In CHO M2 cell membranes, riboflavin and 2′,5′-dideoxyadenosine inhibited Mn^{2+}-, isoproterenol-, and forskolin-stimulated adenylate cyclase, and both were most efficacious vs. Mn^{2+}-stimulated cyclase (Fig. 8).

Riboflavin was remarkably ineffective as an inhibitor of adenylate cyclase in human platelet membranes (Fig. 9) and rat cerebral cortical membranes (Fig. 10A), regardless of whether Mn^{2+} or forskolin was used to stimulate the enzyme. 2′,5′-Dideoxyadenosine, however, caused a robust inhibition of cyclase in both platelet and cerebral cortical membranes. Both riboflavin and 2′,5′-dideoxyadenosine did cause marked inhibition of Ca^{2+}-stimulated adenylate cyclase activity in rat cerebral cortical membranes (Fig. 10B).

TABLE 2. Time Dependence of Inhibitory Effects of Riboflavin on Forskolin-Stimulated Adenylyl Cyclase in Membranes of DDT MF-2 and Wild-Type S49 Cells[a]

Cell	Preincubation time (min)	Cyclic AMP formation % inhibition
DDT MF-2	0	17[b]
	5	14
	10	43
	20	59
Wild-type S49	0	24
	5	55
	10	64
	20	79
Cyc^-S49	0	0
	5	38
	10	48
	20	100

[a]After 10 min at 30° without membranes or ATP, the assay mixture, containing 300 μM riboflavin and forskolin (10 μM for DDT MF-2 and wild-type S49 membranes and 100 mM for cyc^-S49 membranes) or containing forskolin alone, was mixed with membranes. The riboflavin-containing assay mixture was then incubated for 0, 5, 10, or 20 min before [^{32}P]ATP + colt ATP were added. The assay was for an additional 10 min. See Methods for assay. Values are from a single experiment in triplicate. The stimulations by forskolin decreased by about 50% after the 20 min preincubation.

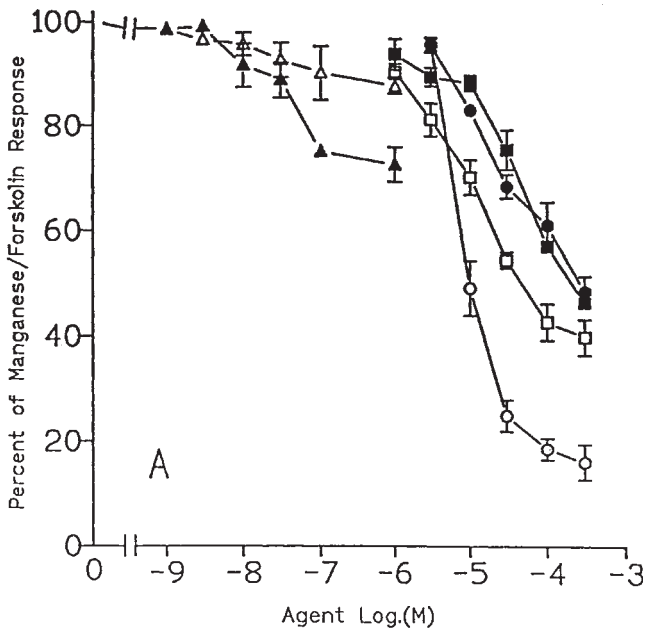
[b]The inhibition by riboflavin was significant less in these experiments than in earlier experiments with DDT MF-2 cells.

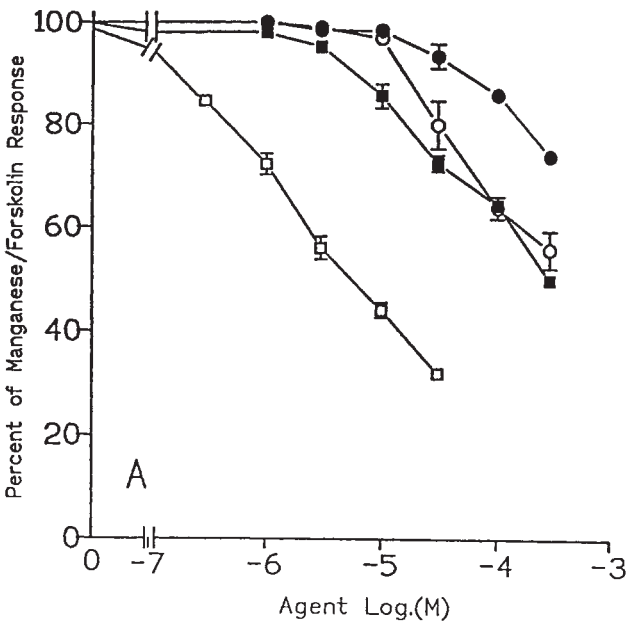
Riboflavin caused only a modest inhibition of forskolin-stimulated adenylate cyclase in membranes from wild-type S49 cells and had no effect in membranes from cyc^-S49 cells (Fig. 11). However, preincubation of either wild-type and cyc^-S49 cell membranes with riboflavin led to a marked inhibition (Table 2). 2′,5′-Dideoxyadenosine markedly inhibited cyclase from wild-type and cyc^-S49 cells.

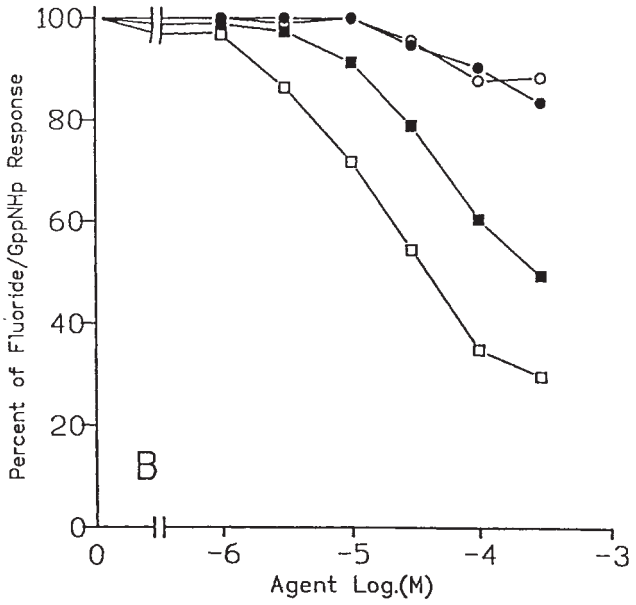
DISCUSSION

Riboflavin is present in the human diet and widely used as a vitamin supplement, where it serves as a precursor of FMN and FAD [Cooperman and Lopez, 1991]. FAD is an essential prosthetic group for such redox enzymes as D-amino acid oxidase, xanthine oxidase, and nitric oxide synthase; the last enzyme is apparently involved in mechanisms of ischemic damage [Iadecola, 1997]. Riboflavin is nearly nontoxic and occurs in only minute amounts in tissues, except in retina. Pharmacologically, it can protect against ischemic damage in both the brain [Hultquist et al., 1993; Betz et al., 1994] and in the heart [Kotegawa et al., 1994].

We now report that at low micromolar concentrations riboflavin binds to A_1-adenosine receptors (Table 1). The lack of an effect of GTP on the affinity of riboflavin for A_1-receptors suggests that riboflavin is an antagonist at such receptors. However, the in-







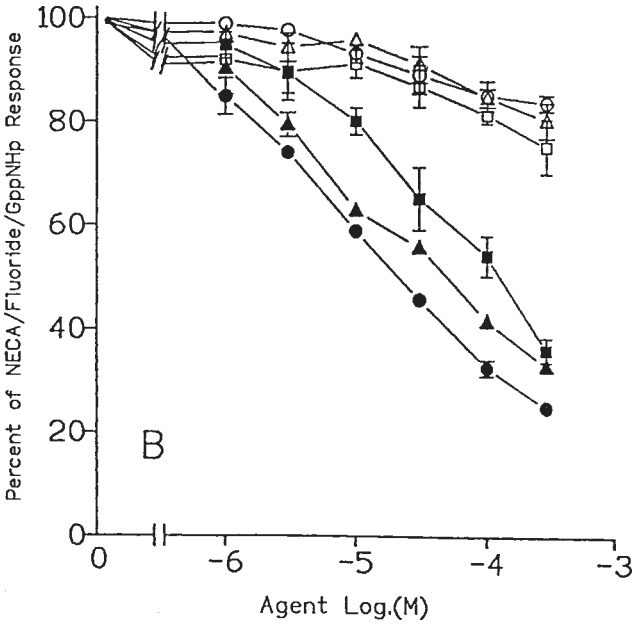


Fig. 6. Inhibitory effects of riboflavin and the P-site inhibitor 2′,5′-dideoxyadenosine on adenylate cyclase activity in DDT MF-2 cell membranes: (**A**) Inhibition of Mn^{2+}-stimulated adenylate cyclase (open symbols) or forskolin-stimulated adenylate cyclase (closed symbols) by N^6-phenylisopropyladenosine (△, ▲), riboflavin (○, ●) or 2′,5′-dideoxyadenosine (□, ■). (**B**) Inhibition of fluoride-stimulated adenylate cyclase (open symbols) or GppNHp-stimulated adenylate cyclase (closed symbols) by riboflavin (○, ●) or 2′,5′-dideoxyadenosine (□, ■). Mn^{2+} concentration was 10 mM, forskolin 10 μM, fluoride 10 mM, and GppNHp 100 μM. The fold-stimulations for the various agents were as follows: Mn^{2+} 3.0-fold, forskolin 9.0-fold, fluoride 8.0-fold, and GppNHp 2.6-fold. See Methods for assay. Values are means ± SEM (n = 3) or are averages from three determinations in a single experiment.

Fig. 7. Inhibitory effects of riboflavin and 2′,5′-dideoxyadenosine on adenylate cyclase in PC12 cell membranes: (**A**) Inhibition of Mn^{2+}-stimulated adenylate cyclase (open symbols) or forskolin-stimulated adenylate cyclase (closed symbols) by riboflavin (○, ●) or 2′,5′-dideoxyadenosine (□, ■). (**B**) Inhibition by riboflavin (open symbols) or 2′,5′-dideoxyadenosine (closed symbols) of N-ethylcarboxamidoadenosine (NECA)-stimulated adenylate cyclase (●, ○), fluoride-stimulated adenylate cyclase (■, □), or GppNHp-stimulated adenylate cyclase (▲, △). Mn^{2+} concentration was 1 mM, forskolin 10 μM, NECA 30 μM, fluoride 10 mM, and GppNHp 100 μM. The stimulations for the various agents were as follows: Mn^{2+} 2.3-fold, forskolin 19-fold, NECA 4.8-fold, fluoride 14-fold, and GppNHp 6.5-fold. See Methods for assay. Values are means ± SEM (n = 3)

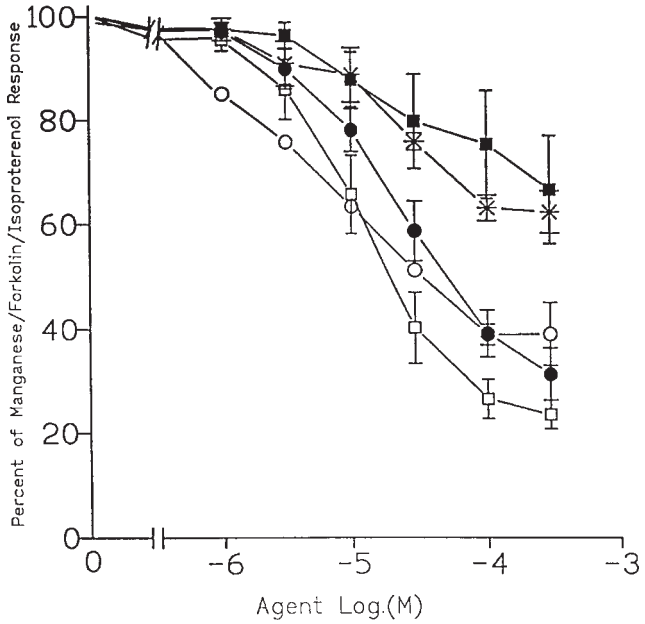


Fig. 8. Inhibitory effects of riboflavin and 2´,5´-dideoxyadenosine on adenylate cyclase in CHO-M2 cell membranes. Inhibition of Mn^{2+}-stimulated adenylate cyclase (open symbols), forskolin-stimulated cyclase (closed symbols), or isoproterenol-stimulated cyclase (asterisks) by riboflavin (●, ○, ∗) or 2´,5´-dideoxyadenosine (□, ■). Mn^{2+} concentration was 10 mM, forskolin 10 μM, and isoproterenol 10 μM. The stimulations for the various agents were: Mn^{2+} 5.2-fold, forskolin 18-fold, and isoproterenol 3.2-fold. See Methods for assay. Values are means ± SEM (n = 3).

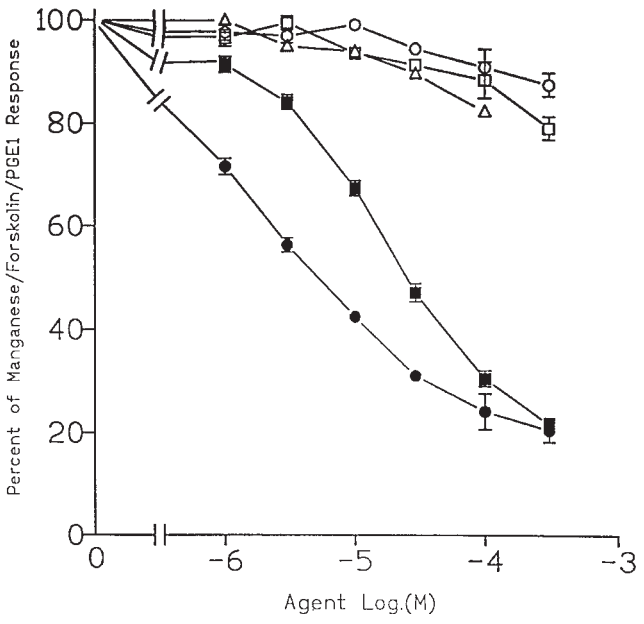


Fig. 9. Inhibitory effects of riboflavin and 2´,5´-dideoxyadenosine on adenylate cyclase in human platelet membranes: Inhibition by riboflavin (open symbols) or 2´,5´-dideoxyadenosine (closed symbols) of Mn^{2+}-stimulated adenylate cyclase (○, ●), forskolin-stimulated adenylate cyclase (□, ■), or prostaglandin E_1 (PGE1)-stimulated adenylate cyclase (△). Mn^{2+} concentration was 1 mM, forskolin 10 μM, and prostaglandin E_1 3 μM. The stimulations for the various agents were as follows: Mn^{2+} 2.3-fold, forskolin 7.0-fold, and prostaglandin E_1 8.0-fold. See Methods for assay. Values are means ± SEM (n = 3).

hibitory effect of riboflavin on adenylate cyclase in two cell types (adipocytes and DDT MF-2 cells) that have A_1-adenosine receptors suggested agonist activity at A_1-adenosine receptors. But the lack of blockade of the inhibitory response by either a xanthine antagonist (Fig. 4) or by inactivation of a requisite G_i protein by pertussis toxin (Fig. 5) argued against such an interpretation. The effects of riboflavin and an A_1-adenosine agonist, R-PIA, were then compared with respect to binding of [^{35}S]GTPγS to G_i-proteins in rat cerebral cortical membranes. Riboflavin acted as an antagonist, blocking the stimulatory effects of the adenosine analog and having no effect alone (Fig. 3A). However, riboflavin also inhibited both basal [^{35}S]GTPγS binding and binding stimulated by fMLP in HL-60 cell membranes (Fig. 3B). Thus, riboflavin appears to inhibit guanyl nucleotide turnover in two different receptor-G-protein systems and may prove to be a general inhibitor of G-protein function. Riboflavin had only very weak effects (K_i > 100 μM) on binding of [^{3}H]CGS 21680 to rat striatal A_{2A}-adenosine receptors [Siddiqi et al., 1996]. Remarkably, binding of [^{125}I]AB-MECA to rat brain A_3-adenosine receptors expressed in COS cells has been reported to be enhanced by riboflavin [Siddiqi et al., 1996].

Further evidence against activation of A_1-adenosine receptors as the mechanism for the inhibitory effect of riboflavin on adenylate cyclase came from results with cells that do not contain A_1-adenosine receptors, namely PC12 cells, CHO cells, and human platelets. In PC12 and CHO cells, riboflavin caused a significant inhibition of adenylate cyclase (Figs. 7, 8).

Certain adenine nucleosides and nucleotides inhibit adenylate cyclase directly by acting at a "P site" on the catalytic moiety [Londos and Wolff, 1997; Nimit et al., 1982; Johnson et al., 1989; Désaubry et al., 1996]. The inhibitory effects of riboflavin on cyclase activity were therefore compared to those of the P-site inhibitor 2´,5´-dideoxy-adenosine. There were both similarities and dissimilarities shown in these comparisons, depending on cell type and on the agent used to stimulate the adenylate cyclase. Both riboflavin and 2´,5´-dideoxyadenosine were more potent inhibitors when cyclase was activated by manganese ions than by forskolin or by a receptor agonist, such as isoproterenol or prostaglandin E_1 (Figs. 6–8). The magnitude of inhibition of cyclase by riboflavin varied greatly among the different cell types. The magnitude of inhibition of adenylate cyclase by 2´,5´-dideoxyadenosine was much

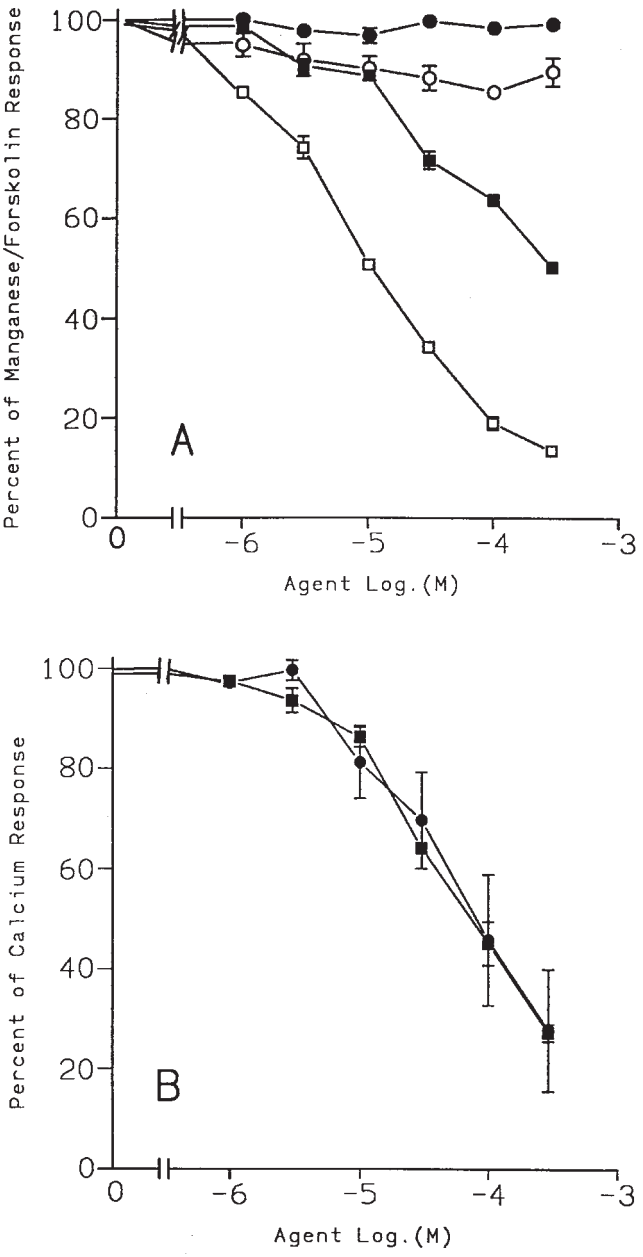


Fig. 10. Inhibitory effects of riboflavin and 2´,5´-dideoxyadenosine on adenylate cyclase in rat cerebral cortical membranes: (**A**) Inhibition of Mn^{2+}-stimulated adenylate cyclase (open symbols) or forskolin-stimulated adenylate cyclase (closed symbols) by riboflavin (●, ○) or 2´,5´-dideoxyadenosine (■, □). (**B**) Inhibition of Ca^{2+}-stimulated adenylate adenylate cyclase in rat cerebral cortical membranes by riboflavin (●) or 2´,5´-dideoxyadenosine (■). Mn^{2+} concentration was 10 mM, forskolin 10 μM, and Ca^{2+} 0.2 mM. The stimulations for the various agents were: Mn^{2+} 3.5-fold, forskolin 4.5-fold, and Ca^{2+} 2.8-fold. See Methods for assay. Values are means ± SEM (n = 3).

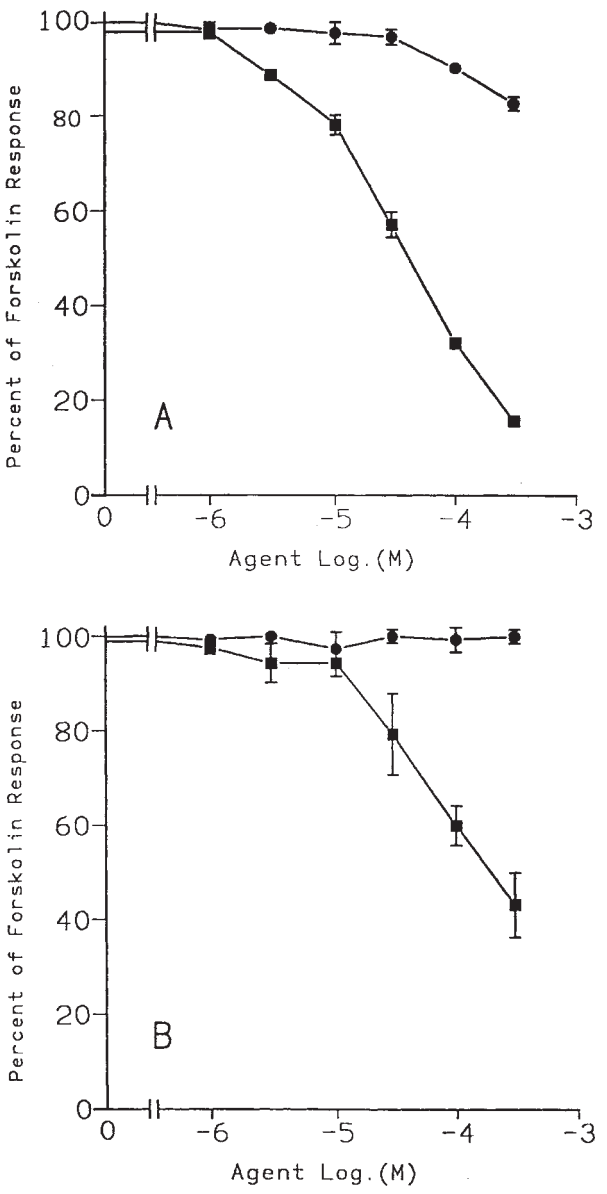


Fig. 11. Inhibitory effects of riboflavin and 2´,5´dideoxyadenosine adenylate cyclase in (**A**) wild-type S49 and (**B**) cyc⁻S49 cell membranes. Inhibition of forskolin-stimulated cyclase by riboflavin (●) or 2´,5´-dideoxyadenosine (■). The stimulation for forskolin (10 μM) was 10-fold in wild-type S49 cell membrane and 3.8-fold in cyc⁻S49 cell membranes. See Methods for assay. Values are means ± SEM (n = 3).

less variable among the different cell types. Adenylate cyclases stimulated by forskolin in human platelet membranes and rat cerebral cortical membranes were relatively insensitive to riboflavin (Figs. 9, 10A). However, the calmodulin-sensitive adenylate cyclase of rat cerebral cortical membranes was markedly inhibited by riboflavin (Fig. 10B). In wild-type S49 cell membranes, riboflavin caused only a slight inhibition, while in cyc⁻ S49 cell membranes, lacking G_s-proteins, there was absolutely no inhibition (Fig. 11A,B). A preincubation of membranes with riboflavin revealed a marked inhibition with wild-type S49 cells, but also revealed a marked inhibition with cyc⁻S49 cells (Table 2). A preincubation

of DDT MF-2 membranes with riboflavin also increased the extent of inhibition.

Inhibitory effects of riboflavin on guanyl nucleotide turnover at G-proteins are clearly demonstrable (Fig. 3A,B), but the involvement of that effect in the inhibition of adenylate cyclase is not resolved. Riboflavin is more effective in inhibiting cyclase that has been activated by agents (forskolin and Mn^{2+}) that act directly on adenylate cyclase than in inhibiting cyclase activated by GppNHp, fluoride, or receptor agonists that act through G_s proteins. Thus, some data suggests an involvement of G-proteins in inhibition of adenylate cyclase by riboflavin, while other data indicates that functional G_s- or G_i-proteins are not necessary.

There are at least eight different adenylate cyclases with distinct pharmacological properties [Désaubry et al., 1996]. However, the distribution of these isoforms is not well characterized. The P-site inhibition of adenylate cyclases by adenine nucleosides and nucleotides is known to occur with all subtypes in a "type-dependent" manner [Désaubry et al., 1996]. Thus, if the inhibition of adenylate cyclase by riboflavin occurs via the P-site, then the interaction with riboflavin would have to be proposed to vary considerably among different subtypes of adenylate cyclase expressed in the different cell types of the present study. The inhibition by 2´,5´-dideoxyadenosine via the P-site would appear relatively consistent. It has been proposed that P-site inhibitors bind to the catalytic site, based on data with one form of adenylate cyclase [Tang et al., 1995].

Another striking difference between riboflavin and 2´,5´-dideoxyadenosine was the lack of inhibition of GppNHp- and fluoride-activated cyclase by riboflavin, contrasting with effective inhibition by 2´,5´-dideoxyadenosine (Figs. 6, 7). It would appear that riboflavin inhibits cyclase, but is ineffective if the cyclase is irreversibly activated by GppNHp or fluoride. Both riboflavin and 2´,5´-dideoxyadenosine inhibit Ca^{2+}-stimulated adenylate cyclase in brain membranes (Fig. 10B), a stimulation which does not require GTP [Seamon and Daly, 1982]. It would appear clear that riboflavin does not inhibit adenylate cyclase via the P-site. One alternate hypothesis is that inhibition by riboflavin occurs via a hitherto unsuspected inhibitory site, which may be variable in different subtypes of adenylate cyclase. Another hypothesis, discussed above, is that riboflavin through an interaction with G-proteins causes the inhibition of cyclases. It is well known that levels and hence roles of different G-proteins vary considerably in different cell types. The nature and proportions of the G-proteins and subtypes of adenylate cyclases present in various cell types and tissues would appear to require further investigation with respect to inhibition to riboflavin. The slow time course for inhibition by riboflavin certainly is consonant with an indirect effect, perhaps involving translocations of G_s- or G_i-proteins to adenylate cyclase. Such an indirect slow effect could differ markedly in different cell types, depending on the relative amounts of G-proteins and adenylate cyclase and membrane properties. A third hypothesis is that riboflavin through an interaction with a protein, perhaps not the G_s- or G_i-protein, but instead another G-protein, causes translocation and inhibition of cyclase.

The physiological or pharmacological (in the case of massive vitamin B_2 supplementation) consequences of effects of riboflavin on A_1-adenosine receptors and/or adenylate cyclase are unknown. Riboflavin at 10 mg/kg i.p. caused significant depression of open-field locomotor activity in mice (data not shown). Piloerection and labored breathing also occurred. The locomotor depression was partly reversed by the A_1-adenosine receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine, at 0.25 mg/kg i.p. Thus, such behavioral effects of riboflavin might be thought to involve A_1-adenosine receptors rather than adenylate cyclase inhibition, except that riboflavin appears to be an antagonist rather than an agonist at A_1-receptors. Cerebroprotection by riboflavin [Betz et al., 1994] might involve not only redox effects on heme proteins, but could also involve selective inhibition of adenylate cyclase. A_1-adenosine agonists are inhibitory to adenylate cyclase and are well known to be cerebroprotective [Rudolphi et al., 1992; Von Lubitz et al., 1996].

As a research tool, riboflavin could represent the lead compound for a new class of inhibitors of cyclic AMP-generating systems in intact cells. Certainly, in cells where riboflavin is an effective inhibitor it is similar in potency to the classical P-site inhibitor 2´,5´-dideoxyadenosine [Londos and Wolff, 1977]. Whether or not the inhibition of adenylate cyclase by riboflavin is direct or indirect remains unresolved. Certainly, riboflavin does inhibit turnover of guanyl nucleotides at G_i proteins, but whether such an inhibition is a requisite step remains unresolved. The slow onset of inhibition of adenylate cyclase does suggest an indirect effect. Riboflavin may prove to inhibit adenylate cyclase indirectly through interactions with G-proteins and may in that case provide a research tool for investigating G-protein–adenylate cyclase interactions.

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