

Barbiturates Are Selective Antagonists at A₁ Adenosine Receptors

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Abstract: Barbiturates in pharmacologically relevant concentrations inhibit binding of (*R*)-N⁶-phenylisopropyl[³H]adenosine ([³H]PIA) to solubilized A₁ adenosine receptors in a concentration-dependent, stereospecific, and competitive manner. K_i values are similar to those obtained for membrane-bound receptors and are 31 μM for (±)-5-(1,3-dimethyl)-5-ethylbarbituric acid [(±)-DMBB] and 89 μM for (±)-pentobarbital. Kinetic experiments demonstrate that barbiturates compete directly for the binding site of the receptor. The inhibition of rat striatal adenylate cyclase by unlabelled (*R*)-N⁶-phenylisopropyladenosine [(*R*)-PIA] is antagonized by barbiturates in the same concentrations that inhibit radioligand

binding. The stimulation of adenylate cyclase via A₂ adenosine receptors in membranes from N1E 115 neuroblastoma cells is antagonized only by 10–30 times higher concentrations of barbiturates. It is concluded that barbiturates are selective antagonists at the A₁ receptor subtype. In analogy to the excitatory effects of methylxanthines it is suggested that A₁ adenosine receptor antagonism may convey excitatory properties to barbiturates. **Key Words:** Adenosine receptors—Barbiturates—Adenylate cyclase—Receptor solubilization—[³H]PIA binding—N1E 115 cells. Lohse M. J. et al. Barbiturates are selective antagonists at A₁ adenosine receptors. *J. Neurochem.* 45, 1761–1770 (1985).

The mechanism of action of barbiturates is still unknown. Basically, two hypotheses have been put forward. (1) Barbiturates might interact with the plasma membrane lipids; this might induce changes in plasma membrane volume, in the structure of lipid domains, or in fluidity, which would ultimately impair the function of proteins embedded in the plasma membrane such as enzymes or ionic channels (Seeman, 1972). (2) Alternatively, barbiturates might interact in a specific manner with membrane proteins, in particular with receptors, and might thereby inhibit or stimulate transmembrane signalling (LaBella, 1981). Such a specific interaction has been convincingly demonstrated for the γ-aminobutyric acid (GABA)-receptor complex, where barbiturates appear to interact with the picrotoxin-binding site also in a solubilized and purified receptor preparation (Olsen, 1982). More recently, we have reported an interaction of barbiturates with

A₁ (R_i) adenosine receptors in rat brain (Lohse et al., 1984a).

Adenosine receptors mediate a variety of physiological functions such as vasodilatation, inhibition of platelet aggregation, and lipolysis. They have been subdivided on the basis of their pharmacological profile and their action on adenylate cyclase into inhibitory A₁ or R_i and stimulatory A₂ or R_s receptors (Van Calcar, 1978; Londos et al., 1980). In the CNS, the A₁ subtype has been demonstrated to mediate inhibition of adenylate cyclase activity (Cooper et al., 1980; Ebersolt et al., 1983), of neuronal firing (Schubert et al., 1983), and of neurotransmitter release (Jackisch et al., 1984). Barbiturates inhibit the binding of both agonist and antagonist radioligands to A₁ adenosine receptors (Lohse et al., 1984a). However, the mechanism of this observation remained to be elucidated. We therefore investigated the effects of barbiturates on a solubi-

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Abbreviations used: CHAPS, 3-(3-cholamidopropyl-dimethylammonio)-1-propanesulfonate; DMBB 5-(1,3-dimethyl)-5-ethyl-

barbituric acid; GABA, γ-aminobutyric acid; MCB, *N*-methylcyclobarbituric acid; MPPB, *N*-methyl-5-phenyl-5-propylbarbituric acid; NECA, 5'-*N*-ethylcarboxamidoadenosine; [³H]PIA, (*R*)-N⁶-phenylisopropyl[³H]adenosine; (*R*)-PIA, (*R*)-N⁶-phenylisopropyladenosine; TBPT, *t*-butylbicyclophosphorothionate.

lized receptor preparation and on the regulation of adenylate cyclase via adenosine receptors.

MATERIALS AND METHODS

Materials

(*R*)-*N*⁶-Phenylisopropyl[³H]adenosine (³H]PIA, sp act 49.9 Ci/mmol) and [³²P]phosphoric acid (carrier-free) were from New England Nuclear, Dreieich, F.R.G. Unlabelled (*R*)-*N*⁶-phenylisopropyladenosine [(*R*)-PIA] was a gift from Dr. Stegmeier, Boehringer Mannheim, F.R.G., and 5'-*N*-ethylcarboxamidoadenosine (NECA) was a gift from Prof. Klemm, Byk Gulden, Konstanz, F.R.G. The stereoisomers of *N*-methylcyclobarbituric acid (MCB), *N*-methyl-5-phenyl-5-propyl barbituric acid (MPPB), and hexobarbital were kindly donated by Prof. Knabe, Universität des Saarlandes, Saarbrücken, F.R.G. Stereoisomers of 5-(1,3-dimethyl)-5-ethylbarbituric acid (DMBB) were provided by Dr. Skolnick, NIH, Bethesda, MD, U.S.A. The following drugs were gifts from the respective drug companies: (±)-DMBB (Eli Lilly, Bad Homburg, F.R.G.), (±)-pentobarbital-sodium (Knoll, Ludwigshafen, F.R.G.), amobarbital (Stada, Bad Vilbel, F.R.G.), (±)-hexobarbital and barbituric acid (Bayer, Leverkusen, U.S.A.), and rolipram [4-(3-cyclopentyl-4-methoxyphenyl)-2-pyrrolidone] (Schering, Berlin, F.R.G.). Dulbecco's modified Eagle medium (DMEM), fetal calf serum, and horse serum were from Seromed, Berlin, F.R.G. Cell culture flasks (75 cm² and 150 cm²) were from Corning, Corning, NY, U.S.A. All other reagents were from standard commercial sources and were of analytical or best available grade.

Preparation of rat brain membranes

Membranes from rat brain were prepared with minor modifications as described by Lohse et al. (1984a). In brief, whole forebrains from male Sprague-Dawley rats were placed in 0.32 *M* sucrose at 0°C; the tissue was homogenized with a glass-Teflon potter at 500 rpm for 30 s. For preparation of striatal membranes, striata were dissected out according to Glowinski and Iversen (1966) and treated in the same way. The homogenate was centrifuged at 1,000 *g* for 10 min and the resulting supernatant again centrifuged at 100,000 *g* for 30 min to obtain the combined P₂ and P₃ fractions (Whittaker, 1969). The pellet was resuspended in water and centrifuged at 100,000 *g* for 30 min. After two washing steps using 50 *mM* Tris-HCl, pH 7.4, and the same centrifugation procedure, the membranes were finally resuspended in 50 *mM* Tris-HCl, pH 7.4, frozen in liquid nitrogen, and stored at -80°C. Protein was determined as described by Peterson (1977).

Preparation of N1E 115 neuroblastoma cell membranes

N1E 115 neuroblastoma cells (Amano et al., 1972) were grown in monolayer cultures in DMEM supplemented with 50 U/ml penicillin G, 50 µg/ml streptomycin sulfate, 1 *mM* arginine, 1 *mM* glutamine, and 44 *mM* NaHCO₃, gassed with 9% CO₂ and 91% air. Cells were initially grown in 75-cm² tissue culture flasks in a medium containing 10% fetal calf serum and then transferred to 150-cm² flasks with a medium containing 10% horse serum. The cells were detached from the wall by vigorous shaking and were collected by centrifugation at 1,600 *g* for 20 min. The pellet was resuspended in 10 *mM* Tris-

HCl, 140 *mM* NaCl, pH 7.4, and centrifuged as described above. The cells were then resuspended in 50 *mM* potassium phosphate buffer, pH 7.0, containing 100 *mM* NaCl and 0.5 *mM* EDTA at a cell density of 10⁷-10⁸/ml. Cell lysis was obtained by N₂-cavitation using a pressure of 25 bar for 30 min. EDTA and β-mercaptoethanol were added to give final concentrations of 3 *mM* and 15 *mM*, respectively, before centrifugation for 5 min at 1,000 *g*. The supernatant was centrifuged at 27,000 *g* for 10 min and the resulting pellet washed once with 10 *mM* Tris-HCl, pH 7.4, and 3 *mM* EDTA. Finally, the membranes were resuspended in 10 *mM* Tris-HCl, pH 7.4, frozen in liquid nitrogen, and stored at -80°C.

Solubilization of adenosine receptors

Rat brain membranes were sedimented by centrifugation at 40,000 *g* for 30 min. The pellets were resuspended in 50 *mM* Tris-HCl, pH 7.4, containing 1% of 3-(3-cholamidopropyl-dimethylammonio)-1-propanesulfonate (CHAPS) with the aid of a pipette. After gentle shaking the solution was left on ice for 30 min and was then diluted 1:5 with 50 *mM* Tris-HCl, pH 7.4. Cell membranes were sedimented by centrifugation at 50,000 *g* for 60 min and the supernatant containing the solubilized receptors was used immediately for binding experiments. For control purposes, higher centrifugation speeds (200,000 *g*) were also used; this did not change the amount of binding per milligram protein nor the characteristics of binding, but resulted in a slightly lower yield, probably due to sedimentation of micelles. Therefore the above procedure was adopted routinely. The protein content in the supernatant was determined according to Peterson (1977).

Binding assay

The binding of [³H]PIA to brain membranes was carried out essentially as described by Lohse et al. (1984a). Approximately 100 µg of membrane protein and 1 *nM* radioligand were present in a total incubation volume of 250 µl using 50 *mM* Tris-HCl, pH 7.4, as buffer system; other substances were added as indicated; in all binding assays adenosine deaminase (0.2 U/ml) was added to remove endogenous adenosine (Schwabe and Trost, 1980). The incubation lasted for 45 min at 37°C and for 60 min at 25°C and was terminated by filtration of a 200-µl aliquot through Whatman GF/B glass-fiber filters followed by two 4-ml washes with ice-cold incubation buffer.

[³H]PIA binding to the solubilized receptor was measured in basically the same way. However, the incubation temperature was reduced to 25°C, as the solubilized receptors did not appear to be stable at 37°C; an incubation time of 90 min was needed to ensure equilibrium under all conditions. The glass-fiber filters were soaked in a solution containing 0.3% polyethyleneimine for at least 1 h prior to filtration. This leads to retainment of the solubilized receptors on the filter by electrostatic forces (Bruns et al., 1983a). Without this procedure, no specific binding can be retained on the filter, indicating that the solubilized receptor preparation is essentially free of membrane-bound receptors.

Adenylate cyclase assay

The activity of adenylate cyclase was determined with the method described by Jakobs et al. (1976). [α -³²P]ATP was synthesized according to Walseth and Johnson (1979). The incubation medium contained in a total

volume of 100 μ l: approximately 300,000 cpm [α - 32 P]ATP, 50 μ M unlabelled ATP, 10 μ M GTP, 1 mM MgCl₂, 100 μ M cyclic AMP, 100 μ M EGTA, 100 mM NaCl, 100 μ M rolipram (ZK 62,711), and 0.2 U/ml adenosine deaminase to remove endogenous adenosine. For N1E 115 membranes the cyclic AMP concentration was reduced to 50 μ M, and NaCl was omitted. The incubation time was 10 min at 37°C for striatal membranes and 30 min at 25°C for N1E 115 membranes. Under these conditions the enzyme activity was linear over the entire time range.

Data analysis

Equilibrium binding data were analyzed by nonlinear curve-fitting with the aid of the program SCTFIT (De Lean et al., 1982). Kinetic binding data were fitted by nonlinear regression using the equations and curve-fitting procedures described by Lohse et al. (1984b). Slope factors of inhibition curves (n_H) were calculated from indirect Hill plots. For competitive antagonism, pA_2 values were calculated by linear regression from Schild plots. In other cases, pA_2 values were calculated using the Schild equation $pA_2 = -\log C + \log(CR - 1)$, where C denotes the concentration of the competitor and CR the ratio of either the IC_{50}/EC_{50} values (adenylate cyclase) or the apparent K_D values (radioligand binding) in the presence and absence of the competitor. Binding data are given as specific binding, with the nonspecific binding being defined by the presence of 1 mM theophylline.

RESULTS

Binding to solubilized A₁ adenosine receptors

Barbiturates inhibit the binding of [3 H]PIA to solubilized A₁ receptors in a concentration-dependent manner. This is shown in Fig. 1 for the stereoisomers of DMBB and MPPB. The curves are monophasic with slope factors near unity; the inhibition is stereospecific with the (-)-isomers being more potent than the (+)-isomers. The K_i values for these and other barbiturates are given in Table 1 both for the solubilized and the membrane-bound receptor. In general, there is good agreement between the values for membranes and solubilized re-

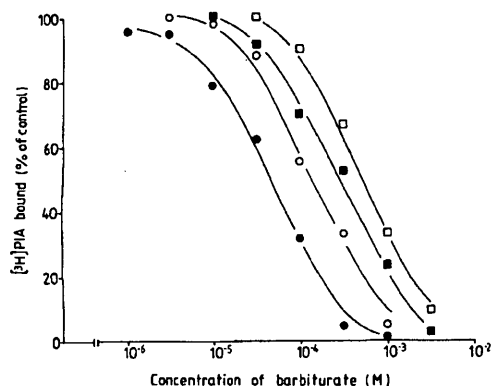


FIG. 1. Inhibition of [3 H]PIA binding to solubilized A₁ adenosine receptors from rat brain by (●), (-)-DMBB; (○), (+)-DMBB; (■), (-)-MPPB; and (□), (+)-MPPB. Data are the means from four experiments.

ceptor, although in many cases there is a tendency toward higher affinities for the solubilized receptor. For all barbiturates the slope factors (n_H) are near unity, suggesting a bimolecular reaction. These data indicate that the inhibition of radioligand binding by barbiturates is due to an interaction with the receptor itself.

The saturation of [3 H]PIA binding to the solubilized receptor in the presence of varying concentrations of (\pm)-DMBB is shown in Fig. 2. Increasing concentrations of DMBB cause considerable flattening of the saturation curve, but the same amount of binding is eventually obtained. The Scatchard plot gives very similar B_{max} values and the apparent K_D values increase with increasing concentrations of DMBB. This characterizes the inhibition of [3 H]PIA binding by barbiturates as a competitive process. The Hill plot using the individual apparent K_D values gives a pA_2 of 4.59. A more accurate assessment can be obtained by simultaneous nonlinear curve-fitting of the untransformed data, which gives a K_i value for (\pm)-DMBB of 26.5 μ M. This agrees well with the value obtained from inhibition curves.

The competitive appearance of the inhibition suggests an interaction of barbiturates with the binding site of the receptor. This assumption is supported by kinetic experiments (Fig. 3). First, the same dissociation curve is seen after addition of a saturating concentration of the A₁ receptor antagonist theophylline and (\pm)-pentobarbital (Fig. 3, left). This indicates that both drugs induce the dissociation of [3 H]PIA from the receptor simply by occupying all binding sites available and thereby preventing reassociation of dissociated radioligand. To exclude any other type of interaction of barbiturates with the receptor, the dissociation was initiated by a saturating concentration of (*R*)-PIA either alone or in the presence of the same concentrations of theophylline and pentobarbital as above. This results in three practically identical dissociation curves (Fig. 3, right); in contrast, the dissociation curve in the presence of 100 μ M GTP, which inhibits agonist binding to A₁ adenosine receptors by an allosteric mechanism via the N_i protein (Rodbell, 1980), is clearly different.

Adenylate cyclase of rat striatum (A₁)

In a previous study we reported that the potency of barbiturates in inhibiting radioligand binding to A₁ adenosine receptors was not affected by guanine nucleotides (Lohse et al., 1984a). This indicates that barbiturates do not induce receptor-N-protein interactions and suggests that they should act as antagonists at the A₁ receptor (Rodbell, 1980). This hypothesis was tested using the adenylate cyclase of rat brain striatal membranes. In these membranes, a 15–25% inhibition of adenylate cyclase activity by adenosine analogues has been observed

TABLE 1. Inhibition of [³H]PIA binding to solubilized A₁ adenosine receptors from rat brain by barbiturates

Barbiturate	Ring substitution			Solubilized receptor		Membrane-bound receptor	
	R ₁	R ₂	R ₃ ^c	K _i (μM)	n _H	K _i (μM)	n _H
(-)-DMBB	H	CH ₂ CH ₃	C*H(CH ₃)CH ₂ CH(CH ₃) ₂	20.9 (17.6–24.8)	1.02 ± 0.04	24.1 (17.6–33.1)	0.94 ± 0.04
(+)-DMBB	H	CH ₂ CH ₃	C*H(CH ₃)CH ₂ CH(CH ₃) ₂	80.1 (60.5–106)	0.93 ± 0.05	81.8 (71.0–94.4)	0.92 ± 0.03
(+)-MCB ^a	CH ₃	CH ₂ CH ₃		168 (153–183)	1.14 ± 0.04	309 (254–374)	0.84 ± 0.02
(-)-MCB ^a	CH ₃	CH ₂ CH ₃		595 (584–606)	1.12 ± 0.03	1,909 (1,395–2,611)	0.99 ± 0.10
(-)-MPPB ^a	CH ₃	CH ₂ CH ₂ CH ₃		179 (150–214)	1.07 ± 0.02	256 (224–292)	0.93 ± 0.08
(+)-MPPB ^a	CH ₃	CH ₂ CH ₂ CH ₃		288 (231–359)	1.13 ± 0.07	522 (326–838)	0.84 ± 0.06
(+)-Hexobarbital ^a	CH ₃	CH ₃		400 (328–488)	0.90 ± 0.07	517 (365–733)	0.89 ± 0.07
(-)-Hexobarbital ^a	CH ₃	CH ₃		773 (731–817)	0.93 ± 0.03	725 (697–754)	0.90 ± 0.01
(±)-DMBB	H	CH ₂ CH ₃	C*H(CH ₃)CH ₂ CH(CH ₃) ₂	30.8 (16.1–58.9)	0.96 ± 0.11	42.9 (27.7–66.6)	0.90 ± 0.09
(±)-Pentobarbital	H	CH ₂ CH ₃	C*H(CH ₃)CH ₂ CH ₂ CH ₃	89.3 (82.9–96.1)	1.13 ± 0.03	107 (64.7–177)	0.92 ± 0.06
Amobarbital	H	CH ₂ CH ₃	CH ₂ CH ₂ CH(CH ₃) ₂	95.4 (87.3–104)	1.05 ± 0.01	142 (132–153)	0.91 ± 0.02
(±)-Thiopental ^b	H	CH ₂ CH ₃	C*H(CH ₃)CH ₂ CH ₂ CH ₃	128 (108–152)	0.89 ± 0.01	169 (161–178)	0.92 ± 0.06
Barbituric acid	H	H	H	>3,000	—	>3,000	—

Given are the K_i values (geometric means and 95% confidence limits) and the slope factors n_H (arithmetic means ± SEM) for solubilized and membrane-bound A₁ receptors determined at 25°C. Data are from three experiments.

^a If R₁ is not H, the C-atom in position 5 is the asymmetric C-atom.

^b In (±)-thiopental, the O-substituent in position 2 is replaced by S.

^c The asymmetric C-atom is marked by an asterisk.

(Cooper et al., 1980; Ebersolt et al., 1983). Barbiturates in concentrations up to 1 mM affected neither basal activity nor the enzyme inhibited by 10 μM (*R*)-PIA; however, in concentrations above 1 mM they cause substantial reductions in both basal and inhibited activity (data not shown). Much lower concentrations of (±)-DMBB are needed to antagonize the inhibition of adenylyl cyclase by (*R*)-PIA (Fig. 4). (*R*)-PIA causes a 25% inhibition of the basal adenylyl cyclase activity; the barbiturate shifts the inhibition curve in a concentration-dependent manner to the right without affecting the degree of inhibition produced by (*R*)-PIA. This confirms the competitive antagonism between (*R*)-PIA and barbiturates at A₁ receptors that was observed in the binding experiments. From the Schild plot (Fig. 4, inset) a pA₂ value of 4.66 for (±)-DMBB can be calculated, corresponding to a K_i value of 21.6 μM. This agrees well with the values obtained from binding studies.

Adenylyl cyclase of N1E 115 neuroblastoma cells (A₂)

In brain membranes the stimulation of adenylyl cyclase, which occurs in the presence of higher concentrations of adenosine analogues, is only relatively small (Ebersolt et al., 1983). In addition, the interpretation is complicated by the presence of in-

hibition via A₁ receptors. Therefore we carried out experiments with membranes obtained from N1E 115 neuroblastoma cells. In these membranes no inhibition of adenylyl cyclase by adenosine analogues could be observed (data not shown). On the other hand, there was a clearcut stimulation of the enzyme by several adenosine analogues, the order of potency being NECA > 2-chloroadenosine > (*R*)-PIA (data not shown). This order of potency has also been shown in other A₂ adenosine receptor systems (Londos et al., 1980). NECA was slightly more efficacious than the other adenosine analogues, a finding that has also been reported for human platelets (Hüttemann et al., 1984). Barbiturates in concentrations <1 mM cause practically no alteration of the stimulation of adenylyl cyclase by NECA. At higher concentrations they inhibit both the basal and the maximally stimulated enzyme activity (Fig. 5, left) in a manner similar to that observed in rat striatal membranes. Using these concentrations (1 mM), a small shift of the stimulation curve to the right can be seen with (±)-DMBB and (±)-pentobarbital (Fig. 5, right). The need of very high concentrations of barbiturates does not allow a detailed analysis using the Schild plot; however, a K_i value can be calculated from the Schild equation. This value is 620 μM for (±)-DMBB and 1.6 mM for (±)-pentobarbital and thus considerably higher

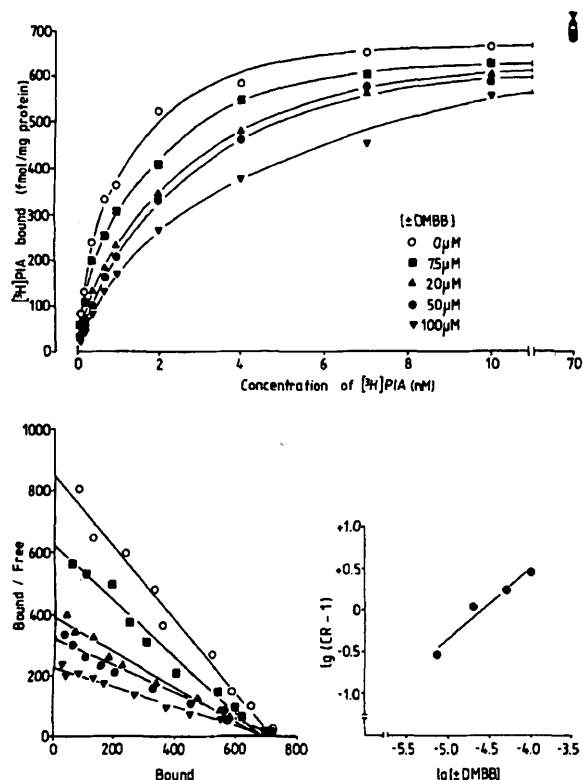


FIG. 2. Saturation of [³H]PIA binding to solubilized A₁ adenosine receptors from rat brain in the presence of increasing concentrations of (±)-DMBB: (○), Control; (■), 7.5 μM; (▲), 20 μM; (●), 50 μM; and (▼), 100 μM. The Scatchard plot (bottom left) gives apparent K_D (and B_{max}) values for [³H]PIA: 0.85 nM (720 fmol/mg protein), 1.11 nM (680 fmol/mg protein), 1.79 nM (680 fmol/mg protein), 2.33 nM (730 fmol/mg protein), and 3.20 nM (720 fmol/mg protein). The Schild plot (bottom right) of the apparent K_D values gives a pA₂ of 4.59 for (±)-DMBB. Simultaneous nonlinear curve fitting gives the following values: B_{max} 1,050 fmol/mg protein, K_D for [³H]PIA 0.87 nM, and K_i for (±)-DMBB 26.5 μM. Data are the means from three experiments.

than the respective values for the A₁ receptor-mediated responses in striatal membranes. To make sure that this difference is not due to a general low affinity of the A₂ receptor from N1E 115 cells for

antagonists, we determined the affinity of the receptor for the classic antagonist theophylline (Fig. 6). Theophylline causes a concentration-dependent shift of the stimulation curve to the right without affecting either basal activity or the degree of stimulation by NECA, indicating competitive antagonism. A pA₂ value of 5.34 for theophylline, corresponding to a K_i value of 4.6 μM, can be calculated from the Schild plot.

Table 2 compares the affinities of barbiturates for A₁ and A₂ receptors as assessed by studies of adenylate cyclase. This demonstrates the selective antagonism of A₁ receptor-mediated effects by barbiturates. The selectivity is almost 30-fold for (±)-DMBB and at least 10-fold for (±)-pentobarbital; however, the latter value should be regarded with some reservation, as the affinity of (±)-pentobarbital for the A₂ receptor cannot be calculated with certainty (Fig. 5). Secondly, Table 2 shows that barbituric acid, which is pharmacologically inactive and does not displace [³H]PIA from the receptor (Table 1), appears also inactive at both A₁ and A₂ receptors when adenylate cyclase activity is studied.

DISCUSSION

Barbiturates have a variety of pronounced effects when given to intact animals or man. However, in spite of a wealth of investigations, attempts to localize and understand their actions have been largely unsuccessful. One of the problems may be that gross electrophysiological recording techniques observe complex phenomena that do not allow a localization of the effects; therefore biochemical studies are needed to identify the structures most sensitive to the action of barbiturates (Smith, 1977).

For a long period, the interaction of barbiturates with plasma membranes has been regarded as the mechanism of action of barbiturates (Dodson and Moss, 1984). However, a number of observations cannot be explained by these theories; in particular, differences in the action of stereoisomers suggest the presence of other mechanisms (Andrews and

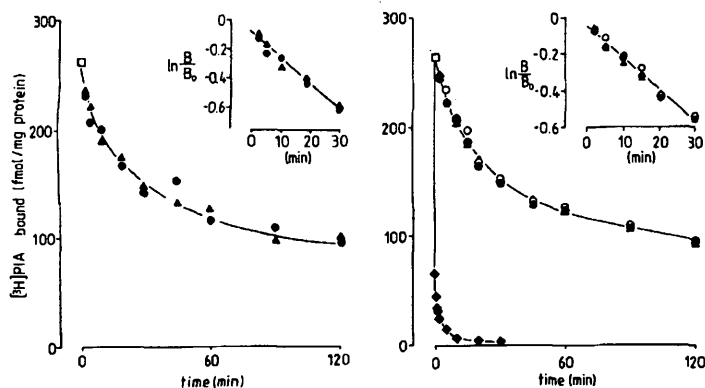


FIG. 3. Dissociation of [³H]PIA from solubilized A₁ adenosine receptors from rat brain. **Left:** After equilibrium had been achieved dissociation was initiated by addition of 1 mM theophylline (▲, k₋₁ 0.016 min⁻¹), or 10 mM (±)-pentobarbital sodium (●, k₋₁ 0.016 min⁻¹). **Right:** The reaction was started by addition of 10 μM (R)-PIA alone (○, k₋₁ 0.017 min⁻¹), 10 μM (R)-PIA + 1 mM theophylline (▲, k₋₁ 0.017 min⁻¹), 10 μM (R)-PIA + 10 mM (±)-pentobarbital sodium (●, k₋₁ 0.018 min⁻¹), or 10 μM (R)-PIA + 100 μM GTP (◆, k₋₁ 5.64 min⁻¹ for the initial phase). The k₋₁ values were obtained by nonlinear curve-fitting. Data are the means of three experiments.

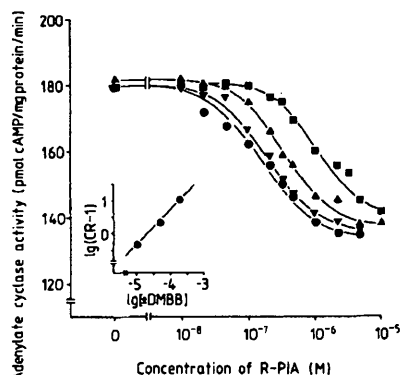


FIG. 4. Inhibition of adenylate cyclase in rat striatal membranes by (*R*)-PIA in the presence of increasing concentrations of (\pm)-DMBB: (●), Control; (▼), 10 μ M; (▲), 50 μ M; (■), 200 μ M. The inset shows the Schild plot of the data using the IC_{50} values; a pA_2 of 4.66 for (\pm)-DMBB is calculated. Data are the means from three experiments.

Mark, 1982). In addition, the interaction of a radiolabelled barbiturate with plasma membranes from rat brain has been shown to be a rather non-specific process that is also shared with barbituric acid (Lohse et al., 1984c). Therefore interactions of barbiturates with membrane proteins must be investigated.

The present study demonstrates interactions of various barbiturates with A_1 adenosine receptors. Equilibrium binding studies with solubilized receptors show a competitive antagonism between barbiturates and [3 H]PIA. The dissociation kinetics are the same, whether a barbiturate or a known A_1 receptor ligand is used to initiate the reaction. This might, by chance, also be the case if the regulation via an allosteric mechanism followed the same time course as the dissociation after occupation of the binding site. However, in this case occupation of the binding site by unlabelled ligand and activation of the hypothetical allosteric site at the same time should result in additive effects. Figure 3 (right) demonstrates this for GTP, which induces uncou-

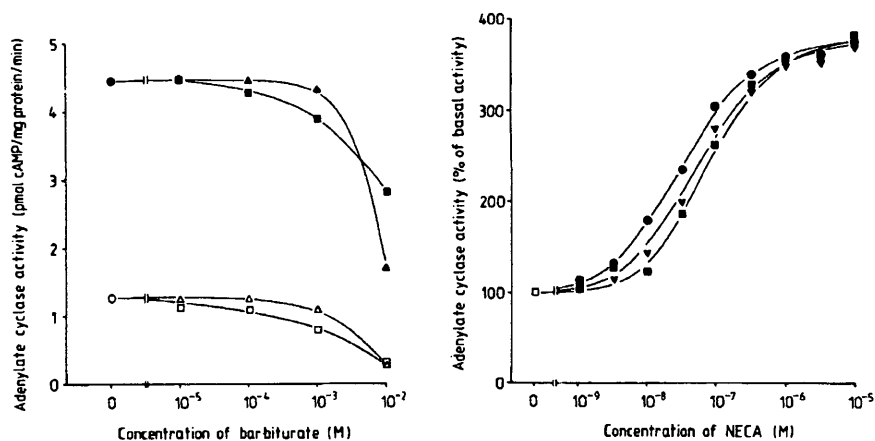
pling of the A_1 receptor and the N_i protein resulting in a decreased affinity of agonists (Goodman et al., 1982; Lohse et al., 1984b); addition of GTP and (*R*)-PIA at the same time leads to a marked acceleration of the dissociation process compared to (*R*)-PIA alone. On the other hand, addition of a barbiturate or of theophylline along with (*R*)-PIA does not cause a deviation of the dissociation curve from the curve with (*R*)-PIA alone. This indicates that all three compounds act at the same site and excludes an allosteric mechanism of the barbiturates.

The reported interaction seems to occur in the pharmacologically relevant concentration range; for example, pentobarbital, which has a K_i value of 90 μ M in inhibiting [3 H]PIA binding, reaches a brain concentration of about 300 μ mol/kg and a serum concentration of about 160 μ M during anesthesia in the rat (Büch et al., 1969). Given a plasma protein binding of about 50% in the rat (Toon and Rowland, 1983) this would result in a free drug concentration of about 80 μ M.

As already suggested from binding experiments investigating the effect of GTP on the affinity of barbiturates (Lohse et al., 1984a), the barbiturates appear to act as antagonists at the A_1 adenosine receptor. Thus, they shift the inhibition of striatal adenylate cyclase by (*R*)-PIA to higher concentrations, and the K_i values calculated from this antagonism agree well with those obtained in binding studies.

Methylxanthines are the most important class of adenosine receptor antagonists. Some analogies in the structure of barbiturates and xanthines can be recognized (Fig. 7). The importance of the ring structure of the barbiturates is underlined by the fact that monoureides such as carbromal do not displace [3 H]PIA from the receptor (Lohse et al., 1984a). Alkyl substituents at C5 are necessary both for the pharmacological activity and for the affinity for the A_1 receptor; these alkyl substituents appear to determine the affinity for the receptor (Table 1). Interestingly, *N*-methylation does not increase the

FIG. 5. Adenylate cyclase in N1E 115 cell membranes. **Left:** Inhibition of basal (open symbols) and stimulated (10 μ M NECA, closed symbols) activity by (\pm)-pentobarbital (Δ , \blacktriangle) and (\pm)-DMBB (\square , \blacksquare). **Right:** Stimulation by NECA: (●), Control; (▼), 1 mM (\pm)-pentobarbital, and (■), 1 mM (\pm)-DMBB. EC_{50} values are 29.7 nM, 48.7 nM, and 78.1 nM, respectively. Data are the means from three experiments.



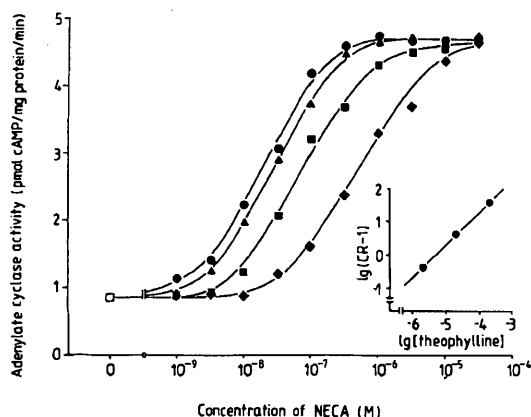


FIG. 6. Antagonism by theophylline of the stimulation of adenylyl cyclase by NECA in N1E 115 cell membranes: (●), Control; (▲), 2 μ M; (■), 20 μ M; and (◆), 200 μ M theophylline. The inset shows the Schild plot of the data using the EC_{50} values; a pA_2 of 5.34 is calculated. Data are the means from two experiments.

affinity of barbiturates; thus, mephobarbital (*N*-methylphenobarbital) has about the same affinity as phenobarbital (K_i values for membrane-bound receptors are 350 μ M and 580 μ M for the (+)- and (-)-isomers of mephobarbital versus 360 μ M for phenobarbital; Table 3 and Lohse et al., 1984a). *N*-Methylation of the xanthine molecule in the corresponding position markedly increases the affinity for adenosine receptors, as evidenced by a 30 times higher affinity of 1-methylxanthine compared to xanthine itself (Bruns et al., 1983b).

In contrast to most methylxanthines, barbiturates appear to be relatively selective for the A_1 receptor. Only pentobarbital and DMBB had measurable effects on the stimulation of adenylyl cyclase via A_2 receptors at concentrations below the level at which marked inhibition of basal activity occurs. Barbiturates in concentrations at or above 1 mM have been reported to alter the fluidity and the phase transition temperatures of the outer half of the plasma membrane; this affects the activity of the receptor-stimulated adenylyl cyclase activity (Houslay et al., 1981). However, the inhibition of

TABLE 2. Barbiturate effects on adenylyl cyclase regulation via adenosine receptors

Barbiturate	Antagonism at		A_1 selectivity ($1/K_i(A_1)/1/K_i(A_2)$)
	A_1 receptor K_i (μ M)	A_2 receptor K_i (μ M)	
(\pm)-DMBB	21.6	620	28.7
(\pm)-Pentobarbital	144	1,600	11.1
Barbituric acid	>3,000	>3,000	—

The values for the A_1 receptor were determined from Schild plots of the antagonism by barbiturates of the inhibition of striatal adenylyl cyclase (as in Fig. 4). Values for the A_2 receptor were determined from the antagonism of stimulation of N1E 115 membrane adenylyl cyclase using the Schild equation (as in Fig. 5).

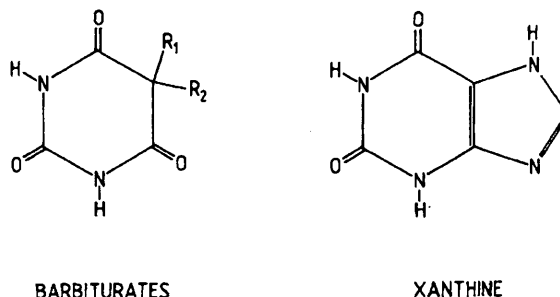


FIG. 7. Structures of barbiturates and xanthine.

basal enzyme activity by high concentrations of barbiturates may also suggest a direct action on adenylyl cyclase.

The small effect of barbiturates on N1E 115 cell membranes contrasts with the high sensitivity of this system compared to other A_2 receptor systems. Thus an EC_{50} of about 30 nM for NECA is seen in these membranes, whereas for other membranes values of 100 nM in liver (Londos et al., 1980), of 500 nM in human platelets (Hüttemann et al., 1984), or >1 μ M in Leydig cell tumor (Londos et al., 1980) have been observed. Similarly, K_i values for theophylline in antagonizing the stimulation by A_2 receptor agonists range from 8 μ M in human platelets (Cusack and Hourani, 1981) to 35 μ M in mouse NS 20 neuroblastoma cells (Blume and Foster, 1975); in N1E 115 cell membranes we measured a K_i value of 4.6 μ M.

Thus the affinities of both agonists and antagonists in these membranes are comparable to those found in intact neuroblastoma cells (Elfman et al., 1984); therefore N1E 115 cell membranes appear to be a particularly useful model for the investigation of A_2 adenosine receptors. Unfortunately, at present no radioligands are available that would allow selective labelling of A_2 receptors (Hüttemann et al., 1984) and thus this method could not be used to investigate the selectivity of barbiturates.

Carbamazepine, an anticonvulsant and antipsychotic drug, has also been found to inhibit radioligand binding to A_1 adenosine receptors (Skerritt et al., 1982; Marangos et al., 1983) and to have a four times lower affinity for A_2 adenosine receptors (Weir et al., 1984). A classification as agonist or antagonist has not been performed, but the comparison of various analogues of carbamazepine suggests that the affinity for A_1 receptors is not related to the anticonvulsant effects of these drugs (Marangos et al., 1983).

Barbiturates also affect other neurotransmitter receptors. An inhibition of radioligand binding to both nicotinic (Miller et al., 1982) and muscarinic (Nordberg and Wahlström, 1984) acetylcholine receptors has been observed. For the nicotinic re-

TABLE 3. Comparison of structure-activity profiles of barbiturates for the GABA receptor-complex and for A_1 adenosine receptors

	GABA receptor		A_1 receptor inhibition of [3 H]PIA binding K_i (μM)
	Inhibition of [35 S]TBPT binding ^a IC_{50} (μM)	Enhancement of [3 H]diazepam binding ^b EC_{50} (μM)	
(-)-DMBB	—	30	24
(±)-DMBB	50	—	36
(±)-Pentobarbital	90	130	92
Amobarbital	—	300	133
(±)-Hexobarbital	220	—	431
Phenobarbital	480	>200	360

Values for the A_1 receptor refer to the membrane-bound receptor measured at 37°C and are the means from three experiments.

^a From Ramanjaneyulu and Ticku (1984).

^b From Leeb-Lundberg and Olsen (1982).

ceptor an allosteric mechanism has been made plausible (Dodson and Miller, 1983).

At the GABA-receptor complex, barbiturates appear to bind to the picrotoxinin/*t*-butylbicyclopheosphorothionate (TBPT) binding site (Ticku and Olsen, 1978; Ramanjaneyulu and Ticku, 1984). The binding of barbiturates to this site leads to enhancement of binding of benzodiazepines and GABA agonists by allosteric mechanisms (Skolnick et al., 1981; Olsen, 1982). This interaction can also be observed in a solubilized and purified receptor preparation (Sigel and Barnard, 1984; Olsen et al., 1984). The potencies of several barbiturates at the GABA-receptor complex and at the A_1 adenosine receptor are given in Table 3. A marked similarity of the structure-activity profile can be seen. This means that, whenever barbiturates interact with the GABA-receptor complex, they also interact with A_1 adenosine receptors. The similarity suggests a relationship between the TBPT binding site and the A_1 receptor which remains to be elucidated.

Methylxanthines are well known CNS stimulants, and it appears that this is due to their antagonism at central A_1 adenosine receptors (Snyder et al., 1981). Thus, it should be expected that the A_1 receptor antagonism by barbiturates conveys CNS stimulant properties to these drugs. Excitatory actions of barbiturates have been observed in many electrophysiological and behavioral studies (Smith, 1977), and a few studies demonstrated increased neurotransmitter release at low doses of barbiturates whereas higher concentrations of barbiturates decreased the release (Ho and Harris, 1981).

Although correlations with these studies are very speculative, a few points deserve mention. The (+)-isomers of DMBB and MPPB are convulsants whereas the (-)-isomers are anesthetics (Downes et al., 1970; Büch et al., 1973). The (-)-isomers are more potent at A_1 adenosine receptors, suggesting that the convulsant properties of the (+)-isomers are not related to A_1 receptor antagonism. In con-

trast, in each case of the stereoisomers used in this study, the more sedative isomer is the more potent at the A_1 receptor (see Downes et al., 1970; Büch et al., 1973; Wahlström and Nordberg, 1984). On the other hand, the more potent anesthetic isomers (-)-MCB and (-)-hexobarbital cause no or less motor excitation than the respective (+)-isomers during anesthesia induction in the rat using an EEG threshold method (Wahlström and Nordberg, 1984). The (-)-isomers, in particular (-)-MCB, are also less potent at A_1 receptors.

Pentobarbital has been shown to be 10 times more potent than phenobarbital in producing a feeling of euphoria (Fraser and Jasinski, 1977). Although pharmacokinetic differences may play a role, it is interesting to note that pentobarbital is several times more potent than phenobarbital at membrane-bound A_1 adenosine receptors (K_i 90 μM versus 360 μM).

Finally, it seems also possible that depressant effects of barbiturates are mediated via the GABA-receptor complex, and excitatory effects via the A_1 adenosine receptor. If this hypothesis is correct, then the ratio of the K_D values at the two receptors might determine the depressant/excitatory properties of a given barbiturate. The validation of this hypothesis requires further investigations.

The present data indicate that antagonism of A_1 adenosine receptors must be considered when the actions of barbiturates are investigated.

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