Naunyn-Schmiedeberg's Arch Pharmacol (1984) 326:69–74

Naunyn-Schmiedeberg's
Archives of
Pharmacology

Interaction of barbiturates with adenosine receptors in rat brain

Martin J. Lohse[1], Volker Lenschow[2], and Ulrich Schwabe[1]

[1] Pharmakologisches Institut der Universität Heidelberg, Im Neuenheimer Feld 366, D-6900 Heidelberg, Federal Republic of Germany
[2] Institut für Pharmakologie und Toxikologie der Universität Bonn, Reuterstraße 2b, D-5300 Bonn 1, Federal Republic of Germany

Summary. The effects of barbiturates on radioligand binding to inhibitory R_i adenosine receptors of rat brain membranes were investigated. Binding of the adenosine receptor agonist (−)N^6-phenylisopropyl[^{3}H]adenosine and the antagonist 1,3-diethyl-8-[^{3}H]phenylxanthine was inhibited by several barbiturates. This inhibition was concentration-dependent and occurred in the range of pharmacologically effective concentrations. Pentobarbital was the most potent of the barbiturates tested with a K_i of 92 μmol/l. The (+)isomers of hexobarbital and mephobarbital were more potent than the respective (−)isomers. Barbituric acid itself did not displace either radioligand in concentrations up to 1 mmol/l. The inhibitory effect of pentobarbital was reversed by a single wash of membranes preincubated with the barbiturate. The presence of pentobarbital caused a decrease of the affinity of the receptor for the antagonist radioligand but did not alter the number of binding sites, suggesting a competitive antagonism. The effects of pentobarbital on radioligand binding to the receptor were not changed by the presence of picrotoxinin nor by the absence of chloride ions. This indicates that they are not mediated via the picrotoxinin binding site. The barbiturates could not be classified as either agonists or antagonists at the R_i adenosine receptor. The presence of GTP did not influence the inhibition of radioligand binding by pentobarbital; this is also observed for antagonists, whereas the affinity of agonists is markedly reduced by GTP. Binding of antagonists to the receptor is enthalpy-driven; the interaction of pentobarbital with the receptor was entropy-driven and the same was true for agonists. Thus, the interaction of pentobarbital with R_i adenosine receptors of rat brain membranes differs from that of both adenosine agonists and antagonists. Our data suggest that R_i adenosine receptors may be involved in the mediation of the effects of barbiturates.

Key words: Adenosine receptors – Barbiturates – Rat brain membranes – Picrotoxinin

Introduction

Adenosine regulates numerous physiological functions via membrane bound receptors (Burnstock and Brown 1980). In addition to peripheral actions, such as coronary vasodilation, inhibition of platelet aggregation and lipolysis, adenosine seems to play an important neuromodulatory role in the central nervous system (Phillis and Wu 1983). It inhibits both transmitter release from nerve endings and neuronal firing (Phillis and Wu 1981; Schubert et al. 1983). Adenosine and its analogues are potent CNS-depressants whereas methylxanthines, which are adenosine receptor antagonists, are potent CNS-stimulants (Snyder et al. 1981). Adenosine receptors seem to be coupled to adenylate cyclase and can be subdivided into inhibitory R_i (A1) and stimulatory R_a (A2) receptors (Van Calker et al. 1978; Londos et al. 1980). Both subtypes have been demonstrated in rat brain (Prémont et al. 1977; Cooper et al. 1980; Ebersolt et al. 1983). Recent evidence suggests that CNS-effects of adenosine are mainly mediated via R_i receptors (Schubert et al. 1983; Jackisch et al. 1983).

The inhibitory R_i receptor has been characterized in the brain of various species by radioligand binding. Agonists such as (−)N^6-phenylisopropyl[^{3}H]adenosine ([^{3}H]PIA)[1], N^6-cyclohexyl[^{3}H]adenosine ([^{3}H]CHA) and 2-chloro-[^{3}H]adenosine or the antagonist 1,3-diethyl-8-[^{3}H]phenylxanthine ([^{3}H]DPX) have been used in binding studies (Bruns et al. 1980; Schwabe and Trost 1980; Williams and Risley 1980). Because of the depressant action of adenosine in the central nervous system we have investigated the interaction of CNS-depressants with radioligand binding to the R_i receptor. We report here that barbiturates displace [^{3}H]PIA as well as [^{3}H]DPX from rat brain synaptosomal membranes at pharmacologically effective concentrations. Part of the results have been presented at the Joint Meeting of the French and German Pharmacological and Toxicological Societies, Freiburg, FRG, 1983 (Lohse et al. 1983).

Methods

Preparation of brain membranes. Crude synaptosomal membranes from rat brain were prepared according to the method described by Whittaker (1969). Male Sprague Dawley rats (150–250 g) were killed be cervical dislocation, the forebrains were quickly removed and immediately placed in 0.32 mol/l sucrose (4° C). The tissue was homogenized in 10 volumes sucrose in a glass/teflon homogenizer (clearing 0.2 mm; 500 rpm for 30 s). The homogenate was centrifuged at 1,000 × g for 10 min to remove the nuclear fraction and the supernatant centrifuged at 30,000 × g for 30 min to give the P_2 fraction. The pellets were resuspended in 10 ml of

Send offprint requests to M. J. Lohse at the above address

1 The abbreviations used are: [^{3}H]PIA, (−)-N^6-phenylisopropyl-[^{3}H]adenosine; [^{3}H]DPX, 1,3-diethyl-8-[^{3}H]phenylxanthine; (−)PIA, (−)-N^6-phenylisopropyladenosine; IBMX, 3-isobutyl-1-methylxanthine; GABA, γ-aminobutyric acid

water and left on ice for 30 min to give synaptosomal membranes. After a final centrifugation step at 48,000 × g for 10 min the membranes were resuspended in 50 mmol/l Tris-HCl buffer, pH 7.4, in a concentration of 6–10 mg protein/ml, frozen in liquid nitrogen and stored at − 18° C until binding assay. Protein was measured according to Lowry et al. (1951).

Binding assay. Measurement of radioligands to synaptosomal membranes was carried out as described previously (Schwabe and Trost 1980). Membranes were diluted in 50 mmol/l Tris-HCl buffer, pH 7.4, to a protein concentration of 1 mg/ml and preincubated for 30 min at 37° C with adenosine deaminase (0.2 U/ml) to remove endogenous adenosine. Binding of [^{3}H]PIA to membranes (100 µg protein per tube) was carried out in 50 mmol/l Tris-HCl buffer, pH 7.4, in a total volume of 1 ml; [^{3}H]PIA was present in a final concentration of 1 nmol/l. Other substances were added as indicated. Incubation was at 37° C for 45 min and was terminated by filtration of a 900 µl aliquot through a Whatman GF/B filter. Filters were immediately washed twice with 5 ml of Tris-HCl buffer (0° C); after addition of 10 ml scintillation cocktail samples were allowed to equilibrate for 12 h before counting in a liquid scintillation counter with an efficiency of approximately 50%.

Binding of [^{3}H]DPX was carried out in essentially the same way, except that the incubation volume was reduced to 250 µl and that the labelled ligand was present in a final concentration of 10 nmol/l; only 2 × 3 ml buffer were used to wash the filter. Incubation time was in general 15 min; in experiments at different incubation temperatures incubation time was 30 min for all temperatures to assure equilibrium binding.

Nonspecific binding was determined in the presence of 10 µmol/l (−)PIA in the case of [^{3}H]PIA binding and of 1 mmol/l theophylline in the case of [^{3}H]DPX binding. It amounted to about 5% and 25%, respectively. Nonspecific binding was not subtracted for data analysis of competition curves.

Data analysis was done with a computer modelling method (SCTFIT) described by De Lean et al. (1982), which allows independent analysis of various parameters as well as a comparison of different models. Slope factors were calculated from indirect Hill plots ("pseudo Hill coefficients"). pA_2 values were calculated according to the Schild equation $pA_2 = -\lg B + \lg(CR - 1)$ where B is the concentration of the competitor and CR the ratio of the apparent K_D values in the presence and absence of the competitor. Thermodynamic parameters were calculated from van't Hoff plots as described by Weiland et al. (1979) using the following equations:

$$\Delta G^\circ = -R \cdot T \cdot \ln K_A, \quad (1)$$

$$\Delta H^\circ = -a \cdot R \quad (2)$$

and

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T \quad (3)$$

where ΔG° (kcal/mol) is the Gibbs free energy change, ΔH° (kcal/mol) the enthalpy change and ΔS° (cal/mol · deg) the entropy change; a is the slope of the van't Hoff plot; R is the gas constant (1.99 cal/mol · deg) and T is the temperature in degrees Kelvin.

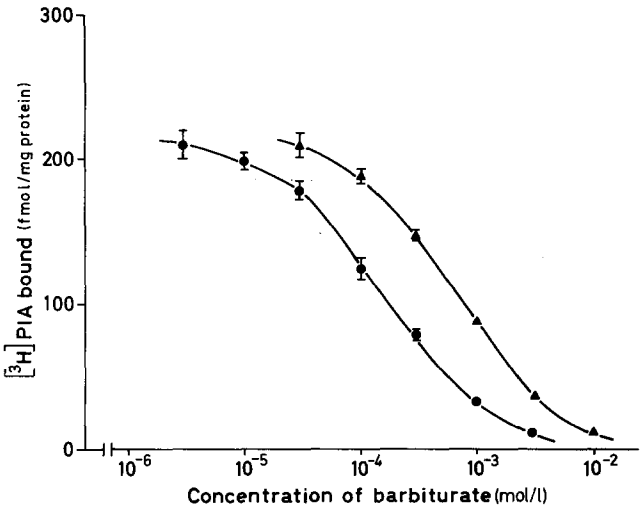

Fig. 1. Displacement of [^{3}H]PIA binding to rat brain membranes by pentobarbital (●——●) and phenobarbital (▲——▲). Binding of [^{3}H]PIA was carried out as described under Methods in the presence of increasing concentrations of the barbiturates (as Na^+-salts). The curves are monophasic with a Hill slope n_H of 0.90 (pentobarbital) and 0.98 (phenobarbital). Data are the mean ± SEM of 5 separate experiments done in duplicate

Materials. (−)-N^6-Phenylisopropyl[^{3}H]adenosine ([^{3}H]PIA, specific activity 49.9 Ci/mmol) and 1,3-diethyl-8-[^{3}H]phenylxanthine ([^{3}H]DPX, specific activity 13.4 Ci/mmol) were obtained from New England Nuclear (Dreieich, FRG). Adenosine deaminase from calf intestine (200 U/mg) and GTP were from Boehringer Mannheim (Mannheim, FRG). 3-Isobutyl-1-methylxanthine, theophylline, γ-aminobutyric acid (GABA), picrotoxinin and barbituric acid were from Sigma (München-Taufkirchen, FRG). The following drugs were gifts of the respective companies: pentobarbital (Desitin, Hamburg, FRG), amobarbital (Stada, Bad Vilbel, FRG), hexobarbital (Bayer, Leverkusen, FRG), thiopental (Byk Gulden, Konstanz, FRG), methohexital (Eli Lilly, Bad Homburg, FRG), oxazepam (Thomae, Biberach, FRG), triazolam (Upjohn, Heppenheim, FRG), naloxone (Endo Laboratories, New York, USA), carbromal (Diabetylin, Blaubeuren, FRG), diphenhydramine (Woelm Pharma, Eschwege, FRG), pentetrazole (Knoll, Ludwigshafen, FRG), bemegride (Nordmark, Uetersen, FRG). (−)-N^6-Phenylisopropyladenosine [(−)PIA] was kindly provided by Dr. K. Stegmeier, Boehringer Mannheim, FRG. The stereoisomers of hexobarbital and mephobarbital were a gift from Prof. J. Knabe, Pharmazeutisches Institut der Universität des Saarlandes, Saarbrücken, FRG. All other chemicals were of analytical or best commercially available grade from standard sources.

Results

The specific binding of [^{3}H]PIA to rat brain membranes is inhibited by various barbiturates in a concentration-dependent manner. Figure 1 shows the displacement of specific [^{3}H]PIA binding by pentobarbital and phenobarbital. Both displacement curves are monophasic and the Hill plots of the data are linear with a slope factor (n_H) of 0.90 for pentobarbital and of 0.98 for phenobarbital.

Table 1. Inhibition of [^{3}H]PIA binding to rat brain membranes. Experiments were carried out as described in the legend to Fig. 1 and K_i values were calculated as described under Methods. The K_D value of [^{3}H]PIA used for calculation was determined in separate saturation experiments and was 1.4 nmol/l. Given are the geometric mean and 95% confidence limits of 5 separate experiments done in duplicate

Compounds	K_i (μmol/l)
Pentobarbital	92 (59–146)
Amobarbital	133 (102–174)
Thiopental	134 (92–196)
Methohexital	344 (339–350)
(±)Hexobarbital	431 (198–718)
(+)Mephobarbital	352 (277–449)
(−)Mephobarbital	578 (487–687)
(+)Hexobarbital	425 (378–478)
(−)Hexobarbital	622 (564–686)
Barbituric acid	>1,000
GABA	>1,000
Picrotoxinin	>1,000
Diphenhydramin	>1,000
Carbromal	>1,000
Bemegride	>1,000
Pentetrazole	>1,000
Oxazepam[a]	> 300
Triazolam[a]	> 300
Haloperidol[b]	> 300

[a] No significant reduction of [^{3}H]PIA binding occurred at 300 μmol/l; higher concentrations were insoluble in the incubation buffer

[b] A 50% reduction of [^{3}H]PIA binding occurred at 1 mmol/l

The K_i values of a number of compounds derived from similar competition experiments are given in Table 1. Pentobarbital is the most potent of the barbiturates tested with a K_i of 92 μmol/l. Thiopental and methohexital, which are more lipid soluble, are less potent. Thus, there is no correlation of lipid solubility and the potency in displacing [^{3}H]PIA binding.

The (+)isomers of mephobarbital and hexobarbital are significantly more potent than the (−)isomers ($P < 0.001$). Barbituric acid itself, which is devoid of CNS-depressant activity, does not displace [^{3}H]PIA binding in concentrations up to 1 mmol/l. A number of centrally active drugs were equally ineffective, including diphenhydramine, carbromal, pentetrazole, bemegride and naloxone. The benzodiazepines oxazepam and triazolam were ineffective in concentrations up to 300 μmol/l, which were maximally obtainable concentrations in aqueous solution. Haloperidol reduced [^{3}H]PIA binding at concentrations far beyond the therapeutic range (1 mmol/l). Drugs acting at the GABA-receptor ionophore complex (GABA, picrotoxinin) were also ineffective in displacing [^{3}H]PIA binding. When [^{3}H]DPX was used as radioligand similar K_i values were obtained (data not shown). Similar K_i values were also obtained using 50 mmol/l Tris-maleate, pH 7.4, as incubation buffer (data not shown).

Next we investigated the question whether this effect of barbiturates is reversible. Figure 2 demonstrates that the inhibition of specific [^{3}H]PIA binding by pentobarbital is almost completely reversed after a single wash with 1 ml of the incubation buffer.

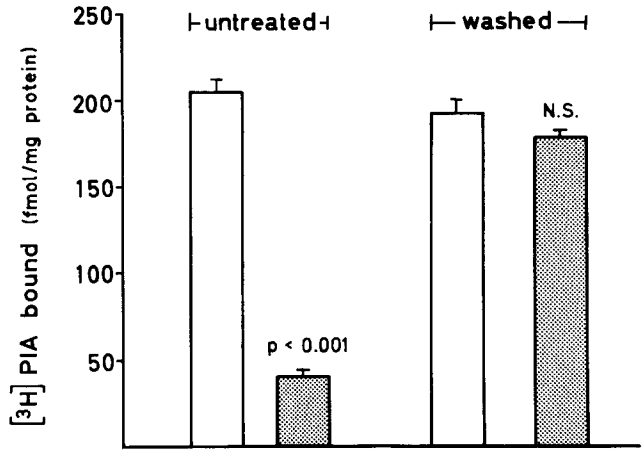

Fig. 2. Reversibility of inhibition of [^{3}H]PIA binding by pentobarbital. Rat brain membranes were preincubated for 15 min at 37° C in the absence (*open bars*) and in the presence of 1 mmol/l pentobarbital (*shaded bars*) in 900 μl of incubation buffer. After preincubation membranes were either left at room temperature ("untreated") or washed once. The washing procedure consisted of a centrifugation step at 12,000 × g for 3 min, aspiration of the supernatant and resuspension of the pellet in 900 μl of incubation buffer. Binding of [^{3}H]PIA was then carried out for all samples at the same time as described under Methods. Data are the mean ± SEM of 3 experiments. *NS*: not significant

The nature of the inhibition of binding to adenosine receptors was further studied by carrying out saturation studies with [^{3}H]DPX in the absence and in the presence of 300 μmol/l pentobarbital, which is a submaximally effective concentration. The results shown in Fig. 3 indicate that the inhibitory effect of pentobarbital can be overcome by increasing concentrations of the radioligand. The Scatchard plot reveals almost identical B_{max} values, but the apparent K_D is about four times higher in the presence of pentobarbital. From the data a pA_2 value of pentobarbital of 4.0 can be calculated; this value agrees well with the K_i value obtained from competition experiments. This suggests a competitive antagonism.

Barbiturates have been shown to interact with the [^{3}H]α-dihydropicrotoxinin binding site, which is part of the GABA-receptor/ionophore complex (Ticku and Olsen 1978). Therefore, it was of interest to see whether blocking this site with high concentrations of picrotoxinin might interfere with the effects of barbiturates on binding to adenosine receptors. Figure 4 shows the displacement of specific [^{3}H]PIA binding in the absence and in the presence of 1 mmol/l of picrotoxinin. At this high concentration picrotoxinin causes a slight reduction of [^{3}H]PIA binding, which is not significant. However, the apparent K_i value of pentobarbital in displacing [^{3}H]PIA binding is not altered.

Considering the possibility of a direct action of barbiturates on R_i receptors we tried to classify them as either agonists or antagonists. The agonist-antagonist properties of drugs can be evaluated by determining the effects of GTP on their ability to compete for [^{3}H]DPX binding (Goodman et al. 1982).

Therefore, competition experiments using [^{3}H]DPX as radioligand were done with the agonist (−)PIA, the antagonist theophylline and pentobarbital in the absence and in the presence of 100 μmol/l GTP (Fig. 5). GTP causes a marked shift of the curve of (−)PIA to higher concentra-

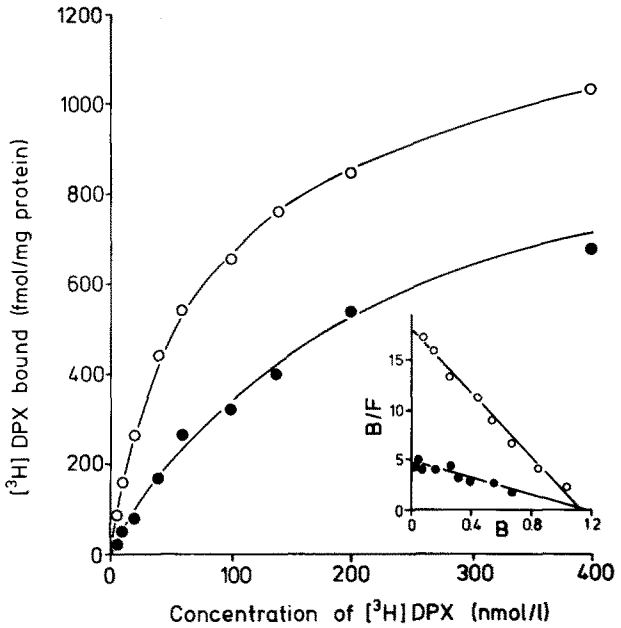

Fig. 3. Effect of pentobarbital (300 µmol/l) on saturation of [^{3}H]DPX binding. Binding of [^{3}H]DPX to rat brain membranes was carried out as described under Methods in the absence (○——○) and in the presence of 300 µmol/l pentobarbital (●——●). In the inset the Scatchard plot of the same data is given (B = bound [^{3}H]DPX in pmol/mg protein; F = free [^{3}H]DPX in µmol/l). Apparent K_D and B_{max} values were calculated by linear regression and are 63 nmol/l and 1.12 pmol/mg protein (control) and 246 nmol/l and 1.15 pmol/mg protein (in the presence of 300 µmol/l pentobarbital). The pA_2 value of pentobarbital is 4.0. Data are the mean of 2 separate experiments done in duplicate

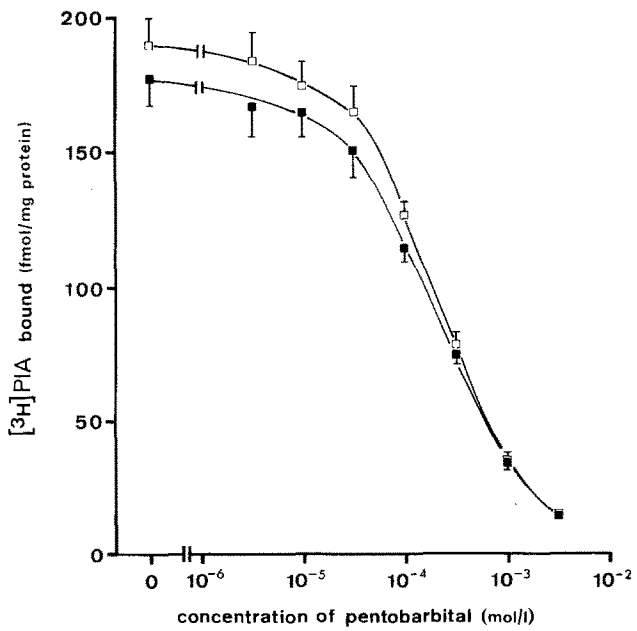

Fig. 4. Effect of picrotoxinin on the displacement of [^{3}H]PIA binding by pentobarbital. Binding of [^{3}H]PIA to rat brain membranes was carried out as described under Methods in the absence (□——□) and presence (■——■) of 1 mmol/l picrotoxin. Apparent K_i values were not significantly different (108 µmol/l vs. 106 µmol/l in control experiments). Data are the mean ± SEM of 5 separate experiments done in duplicate

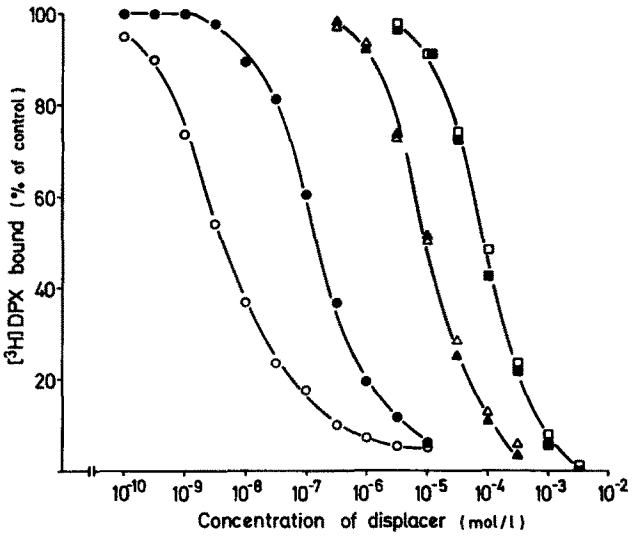

Fig. 5. Effect of GTP on the displacement of [^{3}H]DPX binding by various compounds. Binding of [^{3}H]DPX to rat brain membranes was carried out as described under Methods in the absence (*open symbols*) and in the presence (*closed symbols*) of 100 µmol/l GTP. The displacement curve of (−)PIA (○——○) in the absence of GTP is biphasic indicating the presence of two affinity states. All other curves are monophasic indicating the presence of only one affinity state. The curves of theophylline (△——△) and pentobarbital (□——□) are not significantly altered by the presence of GTP and only one curve is drawn. Data are the mean of five separate experiments done in duplicate

tions. On the other hand, neither the curve of theophylline nor that of pentobarbital are significantly influenced by the presence of GTP.

Agonists and antagonists can further be distinguished by their different thermodynamic characteristics (Weiland et al. 1979; Murphy and Snyder 1982). Therefore, competition experiments with the agonist (−)PIA, the antagonist 3-isobutyl-1-methylxanthine (IBMX) and pentobarbital were carried out at different temperatures. The plot of the data according to van't Hoff (Fig. 6) reveals clear differences between the binding of the agonist and the antagonist: the affinity of (−)PIA is higher at higher temperatures and the contrary is true for the antagonist IBMX. Pentobarbital shows a line with a slightly negative slope which is less steep than that of (−)PIA and is clearly different from the positive slope of IBMX.

The thermodynamic parameters given in Table 2 show that binding of the agonist (−)PIA is completely entropy-driven and binding of the antagonist IBMX is almost exclusively enthalpy-driven. Binding of pentobarbital is entropy-driven with a less unfavourable change of the enthalpy compared to (−)PIA. Thus, there are clear differences in the thermodynamic characteristics of the barbiturate and the R_i receptor antagonist.

Discussion

The mechanism of action of barbiturates is still unknown (Ho and Harris 1981). A number of reports suggest that the GABA receptor/ionophore complex may be involved and barbiturates have been shown to inhibit binding of [^{3}H]-α-dihydropicrotoxinin to a site which is part of this complex

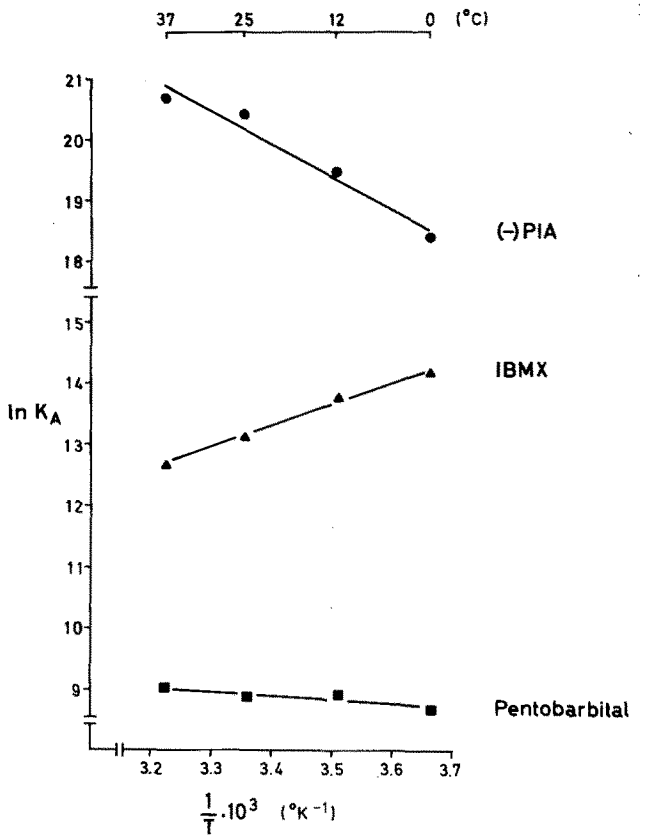

Fig. 6. Van't Hoff plot from displacement of [^{3}H]DPX binding. Experiments were carried out at 0° C, 12° C, 25° C and 37° C and K_i values were calculated as described under Methods. In the case of (−)PIA, K_i values are given for the high affinity state, which is the major component in the absence of GTP. $K_A = 1/K_i$. Correlation coefficients are: $r = -0.97$ [(−)PIA], $r = +0.99$ [IBMX] and $r = -0.75$ [pentobarbital]. Each point is the geometric mean of the K_A values of 3 separate experiments done in duplicate

Table 2. Thermodynamic parameters of binding to rat brain membranes at 37° C. The values of the changes in free energy ($\Delta G°$), enthalpy ($\Delta H°$) and entropy ($\Delta S°$) were calculated from the data shown in Fig. 6 as described under Methods

Compound	($\Delta G°$) (kcal/mol)	($\Delta H°$) (kcal/mol)	($\Delta S°$) (cal/mol · deg)
(−)PIA	− 12.73	10.76	76
IBMX	− 7.81	− 6.91	2.9
Pentobarbital	− 5.86	1.27	23

(Olsen 1982). In addition, a recent study reports modulation of TRH binding to mouse brain by 50 µmol/l pentobarbital (Hirsch 1983).

Our study shows that barbiturates inhibit radioligand binding of agonists and antagonists to R_i adenosine receptors. This effect can be reversed by washing the membranes with incubation buffer after preincubation with a barbiturate. The antagonism between the barbiturates and R_i receptor radioligands seems to be of a competitive nature as indicated by a decrease of the apparent K_D with no change of the B_{max}. The monophasic displacement curves with a Hill slope of about one suggest that a bimolecular reaction takes place.

The K_i value of pentobarbital in competing for [^{3}H]PIA binding is 92 µmol/l. Brain levels of pentobarbital during anaesthesia in the rat have been reported to be 200–300 µmol/kg (Büch et al. 1969); thus, the effect of pentobarbital on radioligand binding to the R_i receptor is well within the range of pharmacologically achieved concentrations. Pentobarbital has been shown to inhibit the release of various transmitters in the CNS in concentrations of 100–500 µmol/l (Ho and Harris 1981).

The (+)isomers of hexobarbital and mephobarbital are more potent than the respective (−)isomers in displacing [^{3}H]PIA. This agrees with the higher anaesthetic potency of (+)hexobarbital compared with the (−)isomer (Wahlström 1966).

Barbiturates are known to interact with the picrotoxinin site of the GABA receptor/ionophore complex (Olsen 1982). Pentobarbital and phenobarbital inhibit binding of [^{3}H]α-dihydropicrotoxinin with an IC_{50} of 50 µmol/l and 400 µmol/l, respectively (Ticku and Olsen 1978). Pentobarbital enhances binding of [^{3}H]GABA and of [^{3}H]diazepam with an EC_{50} of about 100 µmol/l; these effects are dependent on the presence of Cl^--ions and are reversed by picrotoxinin in a competitive manner. The effects of barbiturates on radioligand bindig to R_i adenosine receptors occur in the same concentration range. However, they are not dependent on the presence of Cl^--ions and are not influenced by high concentrations of picrotoxinin. Therefore, barbiturates seem to exert their effects on binding to R_i receptors via a site different from the picrotoxinin binding site.

Another possibility is that barbiturates act at the R_i adenosine receptor itself. However, using two different experiments to distinguish agonists and antagonists we found that barbiturates cannot be classified as either agonists or antagonists at the R_i receptor. The observation that GTP does not influence the displacement of [^{3}H]DPX by pentobarbital could suggest that it is an antagonist; on the other hand the finding that binding of pentobarbital is entropy-driven suggests that it is rather an agonist. Thus, barbiturates do not seem to bind to the receptor itself but may rather exert indirect effects on radioligand binding to the R_i receptor.

In addition to the reports mentioned above which have demonstrated effects of barbiturates on receptors coupled to ionic channels our study shows that barbiturates have also effects on a receptor which is coupled to adenylate cyclase. Houslay et a. (1981) reported that high concentrations of phenobarbital (> 1 mmol/l) modulate glucagon-stimulated adenylate cyclase activity of rat liver plasma membranes. This effect has been interpreted as inhibition of the coupling of the receptor to the GTP regulatory protein. It remains to be clarified whether this effect of barbiturates is related to the effects on radioligand binding to R_i adenosine receptors.

To summarize, our data demonstrate that barbiturates interact with synaptosomal R_i adenosine receptors in the range of pharmacologically effective concentrations. This indicates that effects of barbiturates on the central nervous system may in part be mediated via R_i adenosine receptors.

Acknowledgements. This work has been supported by a grant from the Deutsche Forschungsgemeinschaft (Schw 83/13-1). We appreciate the technical assistance provided by Ms. U. Schwaab.

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Received November 28, 1983/Accepted January 9, 1984