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Effects of barbiturates on A_1adenosine receptors of rat brain

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ABSTRACT

Barbiturates inhibit binding of radioligands to A_1 (R_i) adenosine receptors of rat brain membranes. This inhibition is dose-dependent and stereospecific and occurs in the range of pharmacologically active concentrations. The displacement of radiolabelled A_1antagonists by barbiturates is not modified by GTP, indicating that barbiturates might act as antagonists at this receptor. This action of barbiturates does not seem to be related to the binding of barbiturates to plasma membranes, as the latter process has different characteristics. Barbiturates also inhibit the binding of radioligands to solubilized A_1receptors, and saturation and kinetic experiments suggest that this is due to a competitive antagonism. These results indicate that barbiturates interact with the recognition site of the A_1adenosine receptor.

INTRODUCTION

Adenosine appears to play an important neuromodulatory role in the central nervous system (1). Amongst its most prominent properties is the profound sedation which can be induced by adenosine and its analogues; this effect appears to be mediated by the A_1receptor subtype and conversely A_1receptor antagonists such as the methylxanthines are CNS-stimulants (2). Therefore it seems plausible that also drugs which depress the central nervous system might act via A_1receptors. This hypothesis led us to investigate the effects of barbiturates, one of the major groups of CNS-depressants, on radioligand binding to A_1adenosine receptors of brain membranes.

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METHODS

A P_2-membrane fraction from rat brain was prepared according to Whittaker (3). Binding of [^{3}H](-)N^6-phenylisopropyladenosine ([^{3}H]PIA, 1 nM) and of [^{3}H]diethylphenylxanthine ([^{3}H]DPX, 10 nM) to A_1adenosine receptors was done at 37°C for membranes and at 25°C for solubilized receptors essentially as described (4,5). Bound and free radioligand were separated by rapid filtration. The data were analysed by non-linear curve-fitting as described (6).

RESULTS

Effects of barbiturates on A_1receptor radioligand binding to brain membranes.

Initially the binding of [^{3}H]PIA to a P_2-membrane preparation from rat brain was studied. Barbiturates inhibit the specific binding of [^{3}H]PIA in a dose-dependent manner. Figure 1 shows the inhibition by pentobarbital and phenobarbital. Both curves appear to be monophasic with linear Hill plots and slope factors of 0.90 (pentobarbital) and 0.98 (phenobarbital). Non-linear curve-fitting confirms the homogeneity of the sites.

The K_i-values of other barbiturates together with some CNS-depressants are given in Table 1. DMBB (5-(1,3-dimethylbutyl)-5-

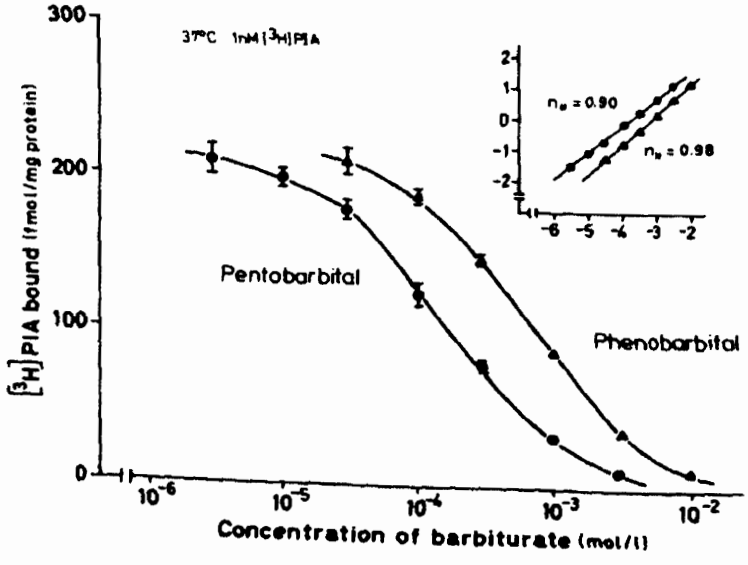

Figure 1 Inhibition of [^{3}H]PIA binding to rat brain membranes by pentobarbital (circles) and phenobarbital (triangles). The inset shows the Hill plot. Data from ref. 5.

ethyl-barbituric acid) is the most potent compound, and the (-)stereoisomer has a K_i-value about 4 times lower than the (+)stereoisomer. Stereospecificity, although to a smaller extent, is also observed for mephobarbital and hexobarbital. In each case the more sedative compound is the more potent in displacing [^{3}H]PIA. Thiopental and methohexital, which are very lipid-soluble, are less potent than pentobarbital; thus, there is no correlation between lipid-solubility and potency in inhibiting binding of [^{3}H]PIA. Barbituric acid which lacks CNS-depressant activity does not inhibit binding of [^{3}H] PIA in concentrations up to 1 mM. Other sedative-hypnotic drugs such as the benzodiazepines triazolam and oxazepam and the antihistamine diphenhydramine are inactive in concentrations up to 1 mM or those maximally obtainable in aqueous solutions.

The affinity of agonists to A_1 receptors is markedly reduced in the presence of GTP, whereas the affinity of antagonists is not altered (7). It is thought that GTP induces the state of low affinity for agonists by uncoupling the receptor and the GTP-regulatory protein N_i (6). This effect is demonstrated in

Table 1 Inhibition of [^{3}H]PIA binding to rat brain membranes.

Compounds	K_i (µmol/l)	confidence limits (µmol/l)
(-)DMBB	24	17 - 33
(+)DMBB	82	71 - 95
(+)Mephobarbital	352	277 - 449
(-)Mephobarbital	578	487 - 687
(+)Hexobarbital	425	378 - 478
(-)Hexobarbital	622	564 - 686
(±)Pentobarbital	92	59 - 146
Amobarbital	133	102 - 174
Thiopental	134	92 - 196
Methohexital	344	339 - 350
Phenobarbital	356	275 - 463
Barbituric acid	>1,000	
Diphenhydramin	>1,000	
Triazolam	> 300	
Oxazepam	> 300	

K_i-values are given as means and 95% confidence limits. Data from ref. 5 and unpublished.

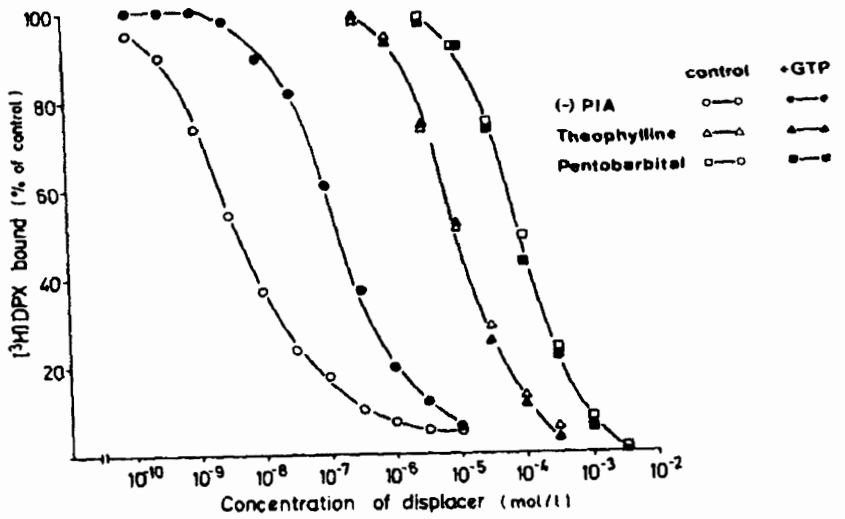

Figure 2 Effect of 100 μM GTP on the displacement of [^{3}H]DPX binding to rat brain membranes by (-)PIA, theophylline and pentobarbital. Data from ref. 5.

Figure 2. When the antagonist radioligand [^{3}H] DPX is used, GTP shifts the inhibition curve of the agonist to higher concentrations while not affecting the curve of the agonist. In addition GTP does not alter the inhibition curve of pentobarbital. This indicates that barbiturates are not agonists at the A_1 receptor.

Binding of barbiturates to brain membranes

The interaction of barbiturates with radioligand binding to A_1 receptors may basically occur at three different sites: 1) at the GTP-regulatory protein N_i which is known to modify the receptor, 2) at the plasma membrane which in turn has influences on the receptor, and 3) at the receptor itself. The first possibility is unlikely because of two findings: firstly barbiturates inhibit [^{3}H]DPX binding which is not subject to influences by N_i, and secondly the effects of barbiturates are the same when GTP is present which uncouples the receptor and N_i.

Effects of barbiturates on plasma membranes have been claimed to be responsible for their pharmacological activity (8). Therefore we have investigated the binding of radiolabelled barbiturates to plasma membranes. [^{3}H] Phenobarbital binds in a saturable manner to a P_2-fraction from rat brain (Figure 3). However, the very high B_{max}-value of 2.7 nmol/mg protein

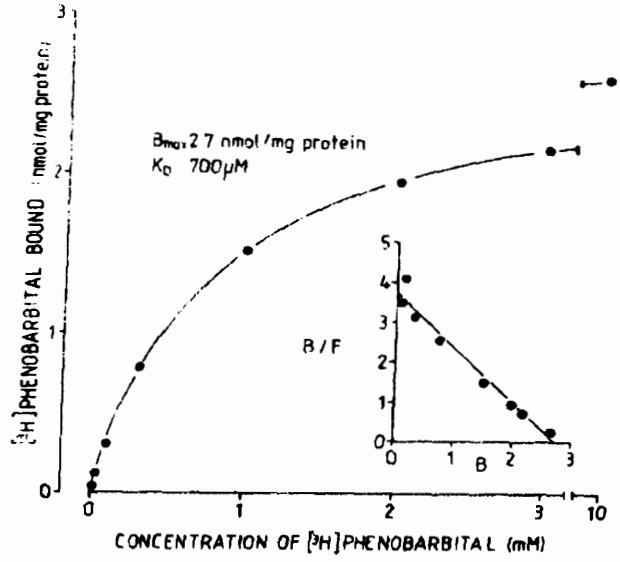

Figure 3 Saturation isotherm of [^{3}H]phenobarbital binding to rat brain membranes. The inset shows the Scatchard plot. Data from ref. 9.

indicates, that the binding site is not a receptor protein. This is confirmed by the fact that the binding is not influenced by temperatures up to 95°C for 20 min. It seems more likely that the binding occurs to membrane lipids. In this case it should be relatively sensitive to detergents. Figure 4 shows that this is indeed true. Various detergents in concentrations as low as 0.1% to 0.2% drastically reduce the binding of [^{3}H]phenobarbital. On the other hand, the binding of [^{3}H]PIA to the same membranes at these detergent concentrations is rather increased, and the effects of barbiturates are the same as in the absence of detergents (data not shown). Thus, effects of barbiturates on A_1 receptors can be observed under conditions where the binding of barbiturates to the plasma membrane is abolished.

Therefore the effects of barbiturates on A_1 receptors do not appear to be related to the binding of barbiturates to plasma membranes.

Effects of barbiturates on radioligand binding to solubilized A_1 receptors

Given the observations that barbiturates appear to inhibit radioligand binding to A_1 receptors neither via N_i nor via the plasma membrane, we considered the possibility that they

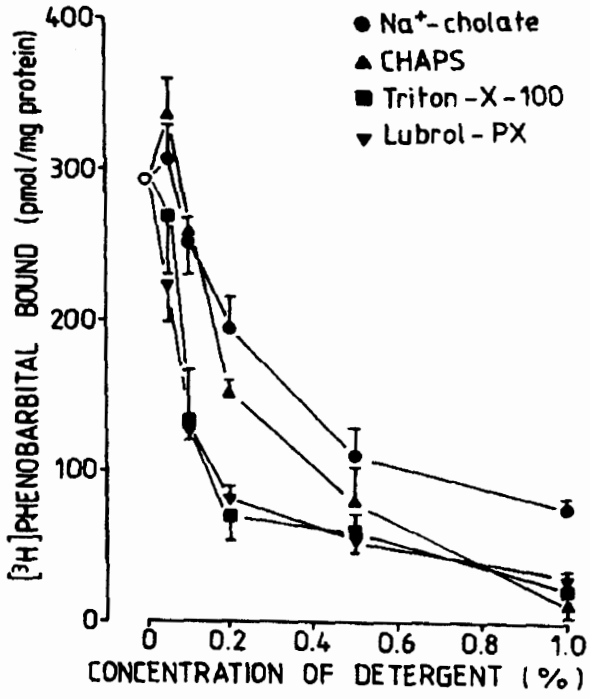

Figure 4 Effects of detergents on [^{3}H]phenobarbital binding to rat brain membranes. Data from ref. 9.

might interact with the receptor itself. Receptors from rat brain P_2-membranes were solubilized with 1% CHAPS (3-((3-cholamidopropyl)-dimethylammonio)-propanesulfonate) and separated from plasma membranes by high speed centrifugation. Binding of

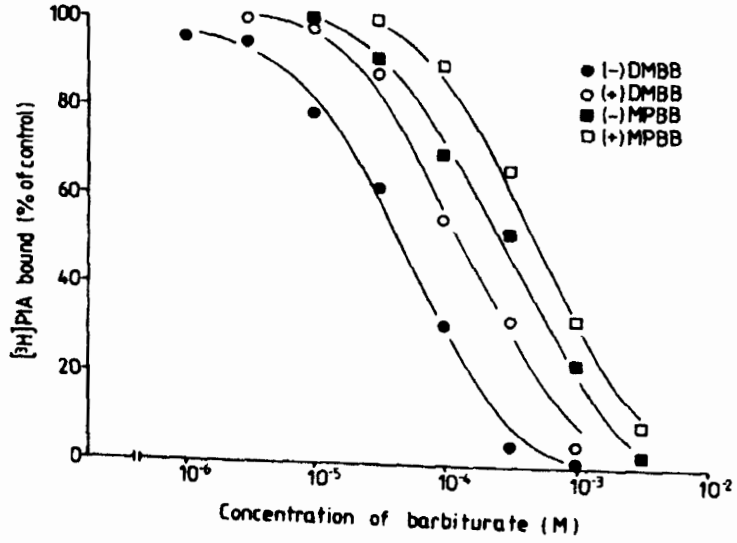

Figure 5 Inhibition of [^{3}H]PIA binding to solubilized A_1 receptors from rat brain by the stereoisomers of DMBB and MPPB. Unpublished data.

[^{3}H]PIA to the solubilized receptor is inhibited by barbiturates in the same concentrations as in plasma membranes (Figure 5). Again the (-)stereoisomer of DMBB is about 4 times more potent than the (+)stereoisomer (K_i 21 µM vs 80 µM), and a slightly lesser degree of stereospecificity is observed for MPPB (N-methyl-5-phenyl-5-propylbarbituric acid; K_i 210 µM vs 320 µM).

The saturation isotherm for [^{3}H]PIA binding to the solubilized receptor is markedly shifted to higher concentrations in the presence of 100 µM (±)DMBB, but the same amount of maximal binding is eventually obtained. Analysis by *non*-linear curve-fitting shows a marked decrease of the apparent K_D-value from 0.72 nM to 3.7 nM, but no change of the B_{max}-value (721 vs 743 fmol/mg protein). This indicates competitive antagonism. The Schild equation gives a pA_2-value for (±)DMBB of 4.6 corresponding to a K_i of 25 µM, which agrees well with the values obtained from the inhibition curves.

The competitive antagonism suggests an interaction of barbiturates with the recognition site of the A_1 receptor. If this is the mechanism, then the dissociation of [^{3}H] PIA from the recognition site should be the same after addition of a high excess of either an unlabelled A_1 receptor ligand or barbiturate. Figure 7 demonstrates this for theophylline and pentobarbital.

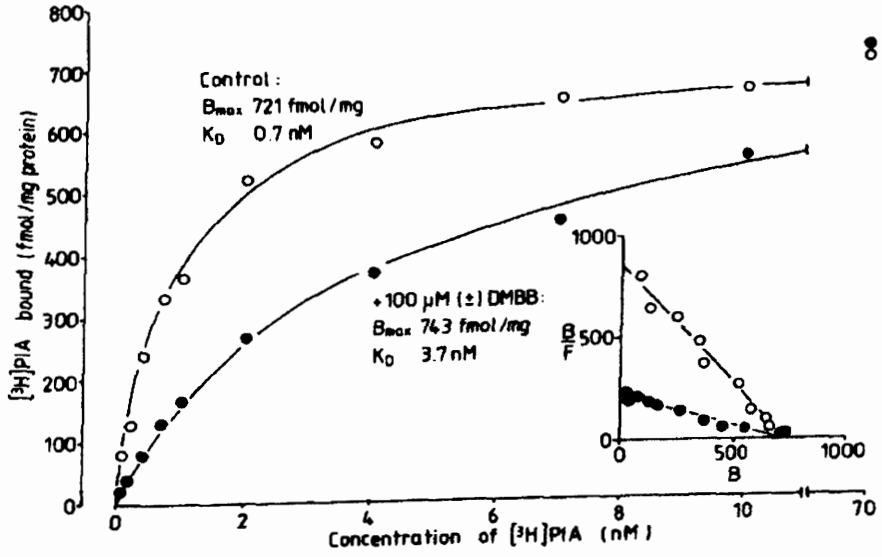

Figure 6 Effect of 100 µM (±)DMBB on the saturation isotherm of [^{3}H]PIA binding to solubilized A_1 receptors from rat brain. The inset shows the Scatchard plot. Unpublished data.

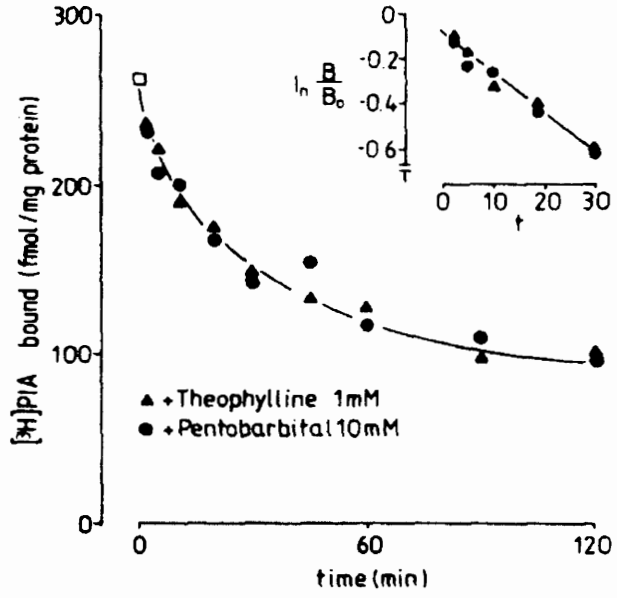

Figure 7 Dissociation of [^{3}H]PIA binding to solubilized A_1 receptors from rat brain. After attainment of equilibrium the dissociation was initiated by addition of 1 mM theophylline or 10 mM pentobarbital. The inset shows the first order plot. Unpublished data.

The dissociation curves are identical for the two compounds. It appears, therefore, that barbiturates interact with the recognition site of the A_1 receptor.

DISCUSSION

Our results indicate that barbiturates interact with A_1 adenosine receptors in the central nervous system. The competitive nature of this interaction and the identity of the dissociation curve after addition of an excess of A_1 receptor ligands and barbiturates suggest that barbiturates act at the recognition site itself. This effect of barbiturates is not modulated by GTP and therefore it appears that barbiturates are antagonists at the A_1 adenosine receptor.

The role of membrane proteins in the mechanism of action of barbiturates and other anaesthetics is still under debate (10). An inhibition of pure luciferase by a variety of anaesthetics underlines the possibility that proteins may be modulated directly and not via alterations of plasma membrane properties

(11). Our study raises the possibility that such an interaction with A_1 receptors may indeed occur in the central nervous system at hypnotic/anaesthetic concentrations. Thus, brain levels of pentobarbital during anaesthesia in the rat have been reported to be 200-300 µmol/kg (12); the K_i-value in inhibiting binding of [^{3}H]PIA is about 100 µM.

Recently two groups reported inhibition of radioligand binding to A_1adenosine receptors by the anticonvulsant carbamazepine (13,14). Although the exact nature of this effect remains to be elucidated it supports the idea that CNS-depressant drugs may exert their effects via A_1adenosine receptors.

ACKNOWLEDGEMENTS

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REFERENCES

1. Phillis, J.W. and Wu,P.H. (1983) in Regulatory functions of adenosine, (ed. Berne, R.M., Rall, T.W. and Rubio, R.) pp 419-437. Martinus Nijhoff Publishers, The Hague, Boston, London.
2. Snyder, S.H., Katims, J.J., Annau, Z., Bruns, R.F. and Daly, J.W. (1981) Proc. Natl. Acad. Sci. USA 78, 3260-3264.
3. Whittaker, V.P. (1969) in Handbook of neurochemistry, vol. 2, (ed. Lajtha, A.) pp 327-364. Plenum Press, New York.
4. Schwabe, U. and Trost, T. (1980) Naunyn-Schmiedeberg's Arch. Pharmacol. 313, 179-187.
5. Lohse, M.J., Lenschow, V. and Schwabe, U. (1984) Naunyn-Schmiedeberg's Arch. Pharmacol. 326, 69-74.
6. Lohse, M.J., Lenschow, V. and Schwabe, U. (1984) Mol. Pharmacol. 26, 1-9.
7. Goodman, R.R., Cooper, M.J., Gavish, M. and Snyder, S.H. (1982) Mol. Pharmacol. 21, 329-335.
8. Seeman, P. (1972) Pharmacol. Rev. 24, 583-655.
9. Lohse, M.J., Klotz, K.-N., Ukena, D. and Schwabe, U. (1984) Neurosci. Lett. (in press).

10.LaBella, F.S. (1980) Can. J. Physiol. Pharmacol. 59, 432-442.
11.Franks, N.P. and Lieb, W.R. (1984) Nature 310, 599-601.
12.Büch, H., Grund, W., Buzello, W. and Rummel, W. (1969) Biochem. Pharmacol. 18, 1005-1009.
13.Skerrit,J.H., Davies, L.P. and Johnston, G.A.R. (1982) Eur. J. Pharmacol. 82, 195-197.
14.Weir, R.L., Padgett, W., Daly, J.W. and Anderson, S.M. (1984) Epilepsia 25, 492-498.

DISCUSSION

Fredholm:
Isn't it possible that you could have some kind of an agonist state which is not necessarily linked to the GTP-binding protein and therefore dissociation is not influenced by GTP ?

Lohse:
When you think about agonists, we are always having adenylate cyclase in our mind and then you would always see that. But of course, there are possible other ways of adenosine mechanisms which we have not investigated.

Fredholm:
Wouldn't that be nice if you had two different types of conformations and barbiturates could only act on one type of conformation?

Daly:
Have you looked at the stimulatory Ra-receptor? I am thinking on adenylate cyclase activation.

Lohse:
We are just studying this.

Dunwiddie:
At least two other classes of compounds have been reported to have barbiturate-like effects. Etomidate is one, it is an anaesthetic drug. It fasciltates GABA -ergic inhibition in brain slices. So, it acts like barbiturates and it also regulates the GABA-binding. I was wondering whether you have looked at other classes of compounds which look biochemically and functionally like barbiturates in order to see whether they are adenosine antagonists or agonists.

Lohse:
Yes, we have also looked at etomidate . The problem is that it is active with a Ki value of about 100 micromolar which is relatively high compared to its anaesthetic concentration, which is in the range of 6 micromolar. So, that appears not too relevant. And secondly, the stereoisomeres of etomidate have very different pharmacological activities. I don't remember which one is anaesthetic; one of them does absolutely nothing. But in our system they do practically the same. So, I don't think that this mechanism is particularly relevant for etomidate.

Phillis:
Your data are somewhat reminiscent of the data on carbamazepine which has been reported to be an adenosine receptor ligand (Skerrit et al. Epilepsia, 24,643, 1983). But in Marangos' hands (Europ. J. Pharmacol. 83, 175, 1983) it is an adenosine receptor antagonist, again based on binding studies which are very similar to the ones which you have done.

Lohse:
Yes, that is very similar, indeed.