19. Heikkila RE, Kindt MV, Sonsalla PK, Giovanni A, Youngster SK, McKeown KA and Singer TP, Importance of monoamine oxidase A in the bioactivation of neurotoxic analogs of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc Natl Acad Sci USA* **85**: 6172–6176, 1988.
20. Johannessen JN, Savit JM, Markey CJ, Bacon JP, Weisz A, Hanselman DS and Markey SP, The development of amine substituted analogs of MPTP as unique tools for the study of MPTP toxicity and Parkinson's disease. *Life Sci* **40**: 697–704, 1987.
21. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
22. Michel PP, Dandapani BK, Sanchez-Ramos J, Efange S, Pressman BC and Hefti F, Toxic effects of potential environmental neurotoxins related to 1-methyl-4-phenylpyridinium on cultured rat dopaminergic neurons. *J Pharmacol Exp Ther* **248**: 842–850, 1989.
23. Michel PP, Dandapani BK, Efange SMN and Hefti F, Potential environmental neurotoxins related to 1-methyl-4-phenylpyridinium: Selective toxicity of 1-methyl-4[4'-acetomidol]phenyl pyridinium and 1-methyl-4-cyclohexyl pyridinium for dopaminergic neurons. *Exp Neurol*, in press.
24. Sundstrom E, Stromberg I, Tetsuyuki T, Olson L and Jonsson G, Studies on the effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on central catecholamine neurons in C57BL/6 mice. Comparison with three other strains of mice. *Brain Res* **405**: 26–38, 1987.
25. Hallman H, Lange J, Olson L, Stromberg I and Jonsson G, *J Neurochem* **44**: 117–127, 1985.
26. Ricaurte GA, Langston JW, DeLanney LE, Irwin I, Peroutka SJ and Forno LS, Fate of nigrostriatal neurons in young mice given 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: A neurochemical and morphological reassessment. *Brain Res* **376**: 117–124, 1986.
27. Johannessen JN, Bacon JP, Markey CJ, Chiueh CC and Markey SP, Striatal DA depletion by an aminated analog of MPTP. *Soc Neurosci Abstr* **12**: 758–1986.

Biochemical Pharmacology, Vol. 40, No. 4, pp. 906–909, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Postmortem stability of the $GABA_A$ receptor complex: a study using rat brain cerebrocortical membranes

(*Received* 2 *January* 1990; *accepted* 19 *April* 1990)

GABA is widely accepted as the major inhibitory neurotransmitter in the mammalian CNS. In addition, the responses to GABA which are mediated by the $GABA_A$ receptor complex are subject to modulation by several centrally acting drugs (for review, see [1]). The distribution and detailed characterization of the $GABA_A$ receptor has been extensively studied in the rat and bovine brain. However, since altered $GABA_A$ receptor function may contribute to the symptoms in several disorders of the CNS such as Parkinson's disease and Huntington's chorea [2, 3], similar detailed characterization of the receptor in human brain is desirable. A prime consideration in human postmortem studies is the stability of the molecular species to be examined. The time from death to autopsy varies considerably in human cases and could well mask any premortem changes associated with age or disease. By far the most convenient way of determining postmortem stability is the indirect method of using animal tissue although this assumes that the stability does not differ significantly between species [4]. These procedures also make the significant assumption that *in vitro* measurements with radioligands can be used to detect premortem changes in functional receptors. In the case of the $GABA_A$ receptor complex it is possible to test the functional state of the receptor complex postmortem to the extent that its putative allosteric properties can be examined using drugs known to modulate the conformation of the receptor complex. The present study reports the effect of postmortem delay on the *in vitro* modulation of the $GABA_A$ receptor by pentobarbitone in rat brain membranes.

Materials and Methods

[Methylamine-^{3}H]muscimol (sp. act. 12.2 Ci/mmol; 107 mCi/mg) was obtained from Amersham International plc (Amersham, Bucks, U.K.). Pentobarbitone (sodium salt), GABA and 5β-pregnan-3α-ol-20-one were obtained from the Sigma Chemical Company (St Louis, MO, U.S.A.). Adult male Sprague–Dawley rats (300–400 g) obtained from the departmental breeding colony within Ninewells Animal Services Unit, University of Dundee, were used. The rats were killed by stunning and cervical dislocation, and the heads were placed in a refrigerator and allowed to cool from 37° to 4° for varying time intervals up to 72 hr to simulate as far as possible the postmortem cooling curve of human cadavers. The brain was then removed and cortices dissected out and stored frozen at −20°. The period between death and refrigeration of the body was examined by leaving rat heads at room temperature after killing for various time intervals up to 6 hr.

Crude preparations of synaptic membranes were prepared from the thawed rat cortices using a procedure similar to that described by Olsen *et al.* [5]. The membranes were suspended in 50 mM tris/citrate buffer pH 7.1 containing 0.1M KCl stored frozen (−20°) prior to assay. On the day preceding the experiment, the membranes were thawed, washed once in tris buffer and dialysed for 20 hr against 1000 vol. of the same buffer.

The binding of [^{3}H]muscimol to the washed synaptic membrane preparation was performed by filtration assay using the protocol of Marangos and Crawley [6]. Aliquots of membrane suspension (equivalent to 0.15–0.25 mg protein) were incubated with the radioligand [^{3}H]muscimol (5 nM, 61 nCi/mL, final concentration), in the absence and presence of 1 mM pentobarbitone in a total vol. of 0.5 mL of 20 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl for 20 min at 0°. Non-displaceable binding was measured in the presence of 100 μM GABA. After incubation, samples were diluted with 4 mL of ice-cold assay buffer and immediately filtered on GF/B filters (Whatman) under suction. Filters were washed three times with 4 mL of buffer, dried and counted for radioactivity in 5 mL Beckman Ready Value scintillation fluid. Protein was determined by the method of Lowry *et al.* [7] with bovine serum albumin as standard.

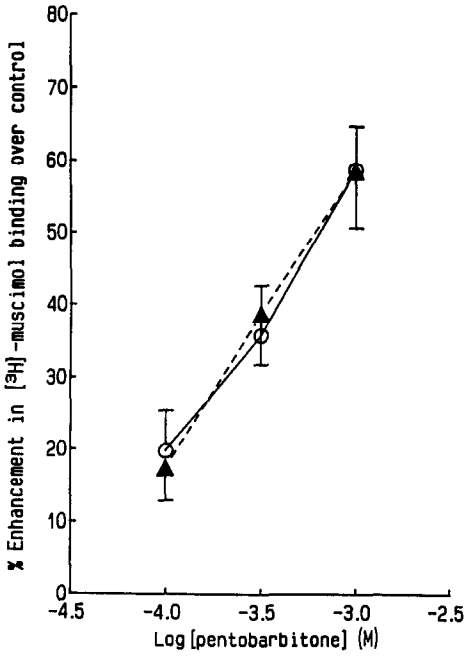

Fig. 1. The enhancement of [^{3}H]muscimol binding evoked by pentobarbitone to crude synaptosomal membranes prepared as described in Materials and Methods using fresh (○) and frozen (▲) brain tissue. Results are the mean ± SE of three experiments.

Data Analysis

The equilibrium dissociation constants (K_d) and receptor densities (B_{max}) were calculated using the 'Ligand' curve-fitting programme of Munson and Rodbard [8]. The effects of pentobarbitone and postmortem delay on [^{3}H]muscimol binding were analysed using an analysis of variance for repeated measures in which [^{3}H]muscimol bound (in presence and absence of pentobarbitone) and postmortem delay were the independent factors analysed. Duncan's test was used for *posthoc* analysis.

Results and Discussion

The effect of freezing brain tissue prior to preparation of crude synaptosomal membranes on the allosteric modulation of [^{3}H]muscimol binding by pentobarbitone as well as on the binding parameters were determined. Parallel binding assays using membranes prepared from fresh and frozen cortices respectively, were carried out using [^{3}H]muscimol concentrations ranging from 1 to 100 nM. The data from both preparations were best fitted to a single site model indicating one population of high affinity binding sites. Freezing significantly increased ($P < 0.05$ compared to control; *t*-test) the affinity but had no effect on the apparent density of [^{3}H]muscimol binding sites in the membrane. The K_d and B_{max} values (mean ± SE, N = 3) were respectively: fresh tissue preparation, 12.79 ± 0.28 nM, 0.79 ± 0.08 pmol/mg protein; frozen tissue preparation, 8.38 ± 0.56 nM, 0.74 ± 0.10 pmol/mg protein. This increase in the affinity of [^{3}H]muscimol binding may be the consequence of facilitated removal of endogenous inhibitors which affects the affinity or it could be due to an effect on the conformation of the receptor protein. Although,

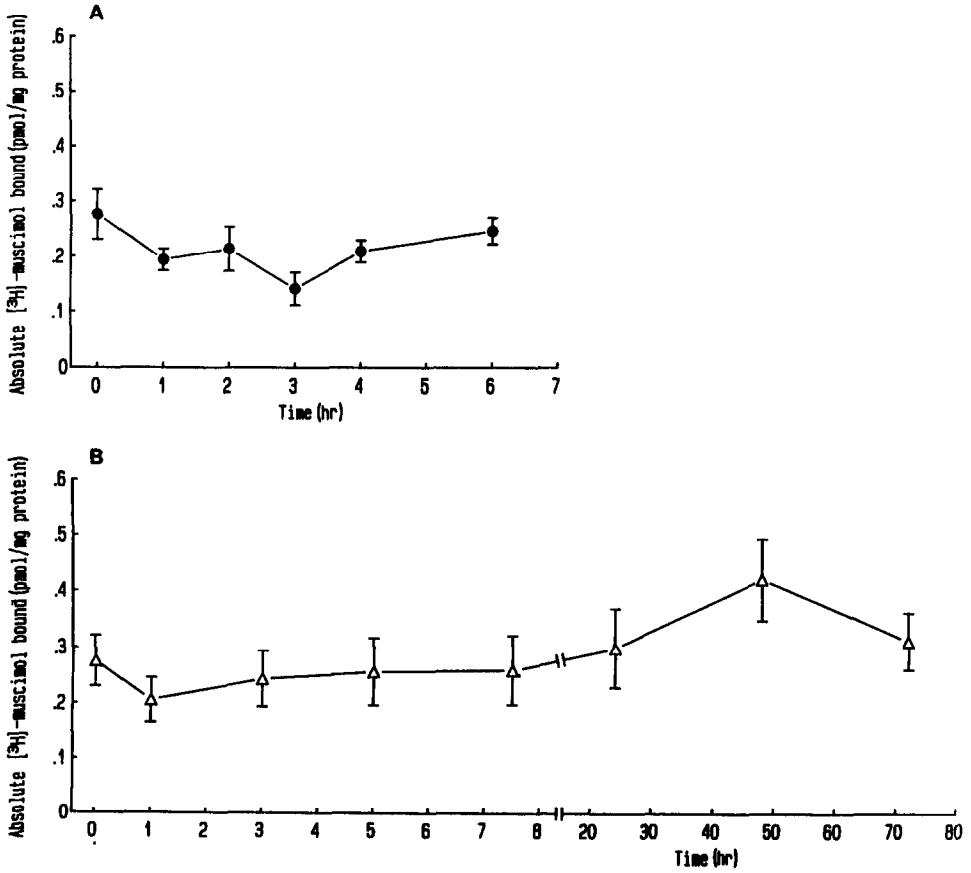

Fig. 2. The effect of postmortem delay at room temperature (A) and 4° (B) on the absolute binding of [^{3}H]muscimol to rat brain membranes. Rat brains were left at room temperature and 4° respectively for various periods after killing before being stored frozen for the preparation of crude synaptosomal membranes. Results are the mean ± SE of three experiments.

following death, there is a rapid rise in GABA concentration in brain tissue, over the longer time course of human studies, concentrations appear to remain constant [9]. The amount of endogenous GABA present is unlikely to interfere with the binding assay since the freezing and dialysis steps carried out should ensure almost complete removal of GABA.

In the presence of pentobarbitone at concentrations of 100 μM and above, the binding of [^{3}H]muscimol was enhanced in a dose-dependent manner (Fig. 1). These results are in agreement with those of other workers who have reported that the enhanced binding appears to reflect an increase in the apparent density of the measured binding sites rather than a change in their affinity for muscimol [10–14]. Parallel binding assays in the presence of pentobarbitone using membranes prepared from fresh and frozen tissue respectively showed that the modulation of muscimol binding was not significantly changed by the freezing process (Fig. 1). The $GABA_A$ receptor complex, therefore, appears to remain functionally intact after freezing. Membranes prepared from rats left at room temperature and at 4° respectively for various time intervals, appeared to show some variation in control [^{3}H]muscimol binding with respect to postmortem delay although this did not reach statistical significance (Fig. 2). The enhancement of [^{3}H]muscimol binding over control evoked by pentobarbitone (1 mM), however, decreased rapidly from 0.16 pmol/mg protein to 0.08 pmol/mg protein (mean of three experiments) during the first hour postmortem at room temperature and thereafter the response remained steady up to 6 hr postmortem (Fig. 3). When compared with the response of membranes prepared from freshly killed rats, the diminished response observed for brain tissue which had been left at room temperature was significant ($F(5,13) = 17.7$; $P < 0.001$; Duncan's test $P < 0.01$) for all the postmortem delay times studied. Postmortem delay at 4° resulted in a less marked decrease in the enhancement of binding over control by pentobarbitone, the enhancement being decreased from 0.21 pmol/mg protein at time zero to 0.11 pmol/mg protein after 7 hr postmortem and thereafter remaining approximately constant up to 72 hr postmortem (Fig. 3). When compared with the response of membranes prepared from freshly killed rats, the diminished response to pentobarbitone observed from brain tissue which had been left at 4° was significant for the following postmortem times studied, 5 hr, 7.5 hr, 48 hr ($F(7, 18) = 2.84$; $P < 0.05$) and 72 hr ($F(7, 18) = 2.84$; $P < 0.01$).

In summary, these studies have shown that the density of $GABA_A$ receptors in membranes prepared from mammalian brain appears to be unaffected by treatments designed to mimic the conditions to which human brain tissue is likely to be exposed postmortem. The ability of the receptor complex to respond to the modulatory effects of pentobarbitone was diminished if the tissue was left at either room temperature or 4° for any length of time. These changes, however, were rapid and predictable and, once they had occurred, the properties of the receptor complex appeared to remain stable for many hours. Thus the data support the hypothesis that it is valid to use membranes prepared from postmortem brain tissue to study the effects of age or disease on the density and allosteric properties of

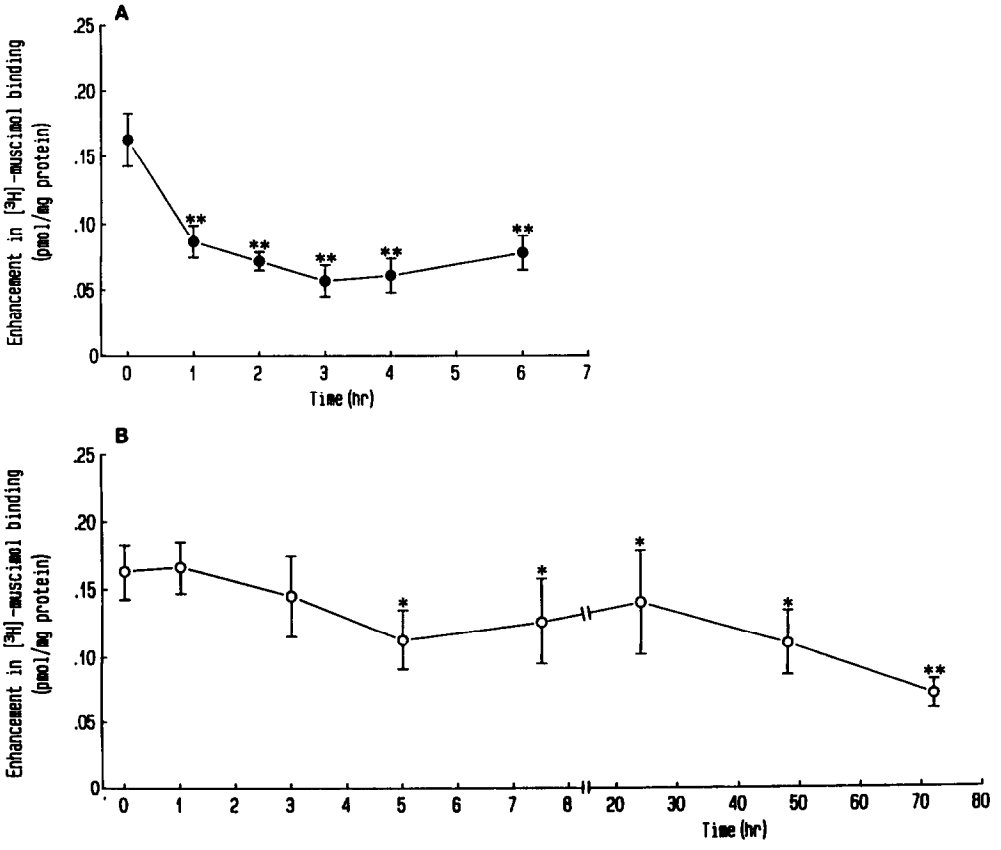

Fig. 3. The effect of postmortem delay at room temperature (A) and 4° (B) on the *in vitro* modulation of [^{3}H]muscimol binding to crude synaptosomal membranes by pentobarbitone. Results are the mean ± SE of three experiments. Significantly different from value at 0 hr: *$P < 0.05$; **$P < 0.01$.

$GABA_A$ receptors although it is necessary to investigate further the molecular basis of the changed response to barbiturates before the hypothesis can be considered fully established.

Department of Pharmacology and Clinical Pharmacology
Ninewells Hospital
University of Dundee
Dundee DD1 9SY, U.K.

JOYCE L. W. YAU
DAVID J. K. BALFOUR*
IAN H. STEVENSON

REFERENCES

1. Bowery NG, Price GW, Hudson AL, Hill DR, Wilkin GP and Turnbull MJ, GABA receptor multiplicity: visualisation of different receptor types in mammalian cns. *Neuropharmacology* **23**: 219–231, 1984.
2. Lloyd KG, Shemen L and Hornykiewicz O, Distribution of high affinity sodium-independent [^{3}H]-gamma-aminobutyric acid ([^{3}H]-GABA) binding in the human brain: alterations in Parkinson's disease. *Brain Res* **127**: 269–278, 1977
3. Lloyd KG, Dreksler S. and Bird ED, Alterations in [^{3}H]-GABA binding in Huntington's Chorea. *Life Sci* **21**: 747-754, 1977
4. Spokes EGS and Koch DJ, Postmortem stability of dopamine, glutamate decarboxylase and choline acetyltransferase in the mouse brain under conditions simulating the handling of human autopsy material. *J Neurochem* **31**: 381–383, 1978.
5. Olsen RW, Snowman AM, and Leeb-Lundberg F, Barbiturates enhance GABA receptor binding in mammalian brain membranes *in vitro*. *Fed Proc* **40**: 309, 1981.
6. Marangos PJ and Crawley JN, Chronic benzodiazepine treatment increases [^{3}H]-muscimol binding in mouse brain. *Neuropharmacology* **21**: 81–84, 1982.
7. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
8. Munson PJ and Rodbard D, Ligand: a versatile computerized approach for characterisation of ligand-binding systems. *Anal Biochem* **107**: 220–239, 1980.
9. Spokes EGS, Garrett NJ and Iversen LL, Differential effects of agonal status on measurements of GABA and glutamate decarboxylase in human postmortem brain tissue from control and Huntington's chorea subjects. *J Neurochem* **33**: 773–778, 1979.
10. Asano T and Ogasawara N, Stimulation of GABA receptor binding by barbiturates. *Eur J Pharmacol* **77**: 355–357, 1982.
11. Olsen RW and Snowman AM, Chloride-dependent enhancement by barbiturates of gamma-aminobutyric acid receptor binding. *J Neurosci* **2**: 1812–1823, 1982.
12. Skerritt JH, Johnston GA, Katsikas T, Tabar J, Nicholson GM and Andrews PR, Actions of pentobarbitone and derivatives with modified 5-butyl substituents on GABA and diazepam binding to rat brain synaptosomal membranes. *Neurochem Res* **8**: 1337–1350, 1983.
13. Supavilai P, Mannonen A, and Karobath M, Modulation of GABA binding sites by CNS depressants and CNS convulsants. *Neurochem Int* **4**: 259–268, 1982.
14. Whittle SR and Turner AJ, Differential effects of sedative and anticonvulsants barbiturate on specific [^{3}H]GABA binding to membrane preparations from rat brain cortex. *Biochem Pharmacol* **31**: 2891–2895, 1982.

* To whom correspondence should be addressed.

Biochemical Pharmacology, Vol. 40, No. 4, pp. 909–911, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Potentiating effects of endothelin on platelet activation induced by epinephrine and ADP

(*Received* 5 *January* 1990; *accepted* 19 *April* 1990)

Endothelin (ET) is a potent vasoconstrictive peptide that has been recently characterized from the supernatant fraction of cultured vascular endothelial cells. It is the only endothelial cell-derived vasoconstrictive substance convincingly identified to date [1]. This property has been demonstrated in a variety of species *in vitro* and *in vivo*.

It would aid hemostatics if ET potentiates platelet function in cooperation with its vasoconstrictive property at the site of endothelial injury. This assumption led us to investigate the potentiating effect of ET on platelet activation induced by epinephrine and ADP.

Materials and Methods

Endothelin-1 (human) was obtained from Peptide Institute Inc. (Osaka, Japan). It was chemically synthesized, and 99% pure, determined with HPLC systems. It contained no other proaggregatory agents. STA_2, a stable thromboxane A_2 analogue, was a generous gift from Ono Pharmaceutical Corp. (Osaka, Japan). Aequorin, 2-7,bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), $DiSC_3(5)$ were obtained from Baxter Travenol (Tokyo, Japan), Dojin Chemistry (Kumamoto, Japan), and Molecular Probes, Inc. (Junction City, OR, U.S.A.), respectively.

Citrate-anticoagulated blood was obtained from healthy human donors who denied having taken any drugs for 14 days preceding the experiment. The blood was centrifuged at 60 *g* for 10 min to obtain platelet-rich plasma (PRP). PRP was adjusted to a concentration of 3×10^8 cells/mL with the corresponding platelet-poor plasma. Five hundred μL of PRP was used to measure aggregation in a Nikou Bioscience Hematracer IV (Tokyo, Japan). Maximal decrease in optical density was defined as maximal aggregation rate (MAR).

In every experiment, subthreshold doses of epinephrine and ADP were determined, which induced a decrease in MAR by less than 40%. Various doses of ET were added to the PRP and the mixture was incubated for 3–5 min at 37°. After the addition of epinephrine or ADP at subthreshold dose, platelet aggregation was measured for 10 min. ET potentiation was determined as "positive" when MAR