Biochemical Pharmacology, Vol. 33, No. 23, pp. 3883–3886, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
Pergamon Press Ltd.

STIMULATION OF SEROTONIN SYNTHESIS IN RAT BRAIN AFTER ANTIEPILEPSIRINE, AN ANTIEPILEPTIC PIPERINE DERIVATIVE

G. Q. LIU,* S. ALGERI, A. CECI, S. GARATTINI, M. GOBBI and S. MURAI†
Istituto di Ricerche Farmacologiche 'Mario Negri', Via Eritrea, 62, 20157 Milano, Italy

(*Received* 25 *July* 1983; *accepted* 6 *July* 1984)

Abstract—Piperine and two of its derivatives, antiepilepsirine (AE or 3,4,methylendioxycynnamoylpiperine) and compound 7448 (*N*-isopropyl 3 (4 chloro-phenyl) propenoylamide) are very effective in stimulating serotonin (5HT) synthesis. AE raises the ratio of free-to-bound tryptophan (TP) in plasma and induces a long-lasting increase of this aminoacid in brain. At the same time in striatum and limbic area it causes a lasting increase in 5 hydroxyindolacetic acid (5HIAA) a 5HT metabolite and to a lesser extent, an increase in the levels of the monoamine itself.

Together with this action on 5HT metabolism we found that AE caused release of ^{3}H-5HT from an *in vitro* synaptosomal preparation. It thus appears that piperine and its derivatives AE and compound 7148 affect the central serotonergic system.

Piperine is considered to be the active principle of *Piper nigrum* L., a plant utilized for the treatment of epilepsy in Chinese popular medicine. Pharmacological studies have indicated that piperine [1] and several of its derivatives [2, 3] protect rats and mice against various kinds of experimental convulsions, including those induced by maximal electroshock, leptazol, picrotoxin and strychnine and potentiate the sedative effect of depressant agents. One of these derivatives, hereafter referred to as AE (antiepilepsirine or 3,4 methylen dioxycynnamoylpiperine), is used clinically for the treatment of grand mal epilepsy [4].

No neurochemical studies have yet been made with piperine derivatives to our knowledge. In view of the fact that other anticonvulsant agents reportedly interfere with neurotransmitters [5–7], we have made a number of experiments to determine whether AE modified serotoninergic and dopaminergic mechanisms. For comparisons, piperine and a derivative, 7448 *N*-isopropyl-3 (4 chlorophenyl)propenoylamide (see chemical structures in Fig. 1) were included in some experiments.

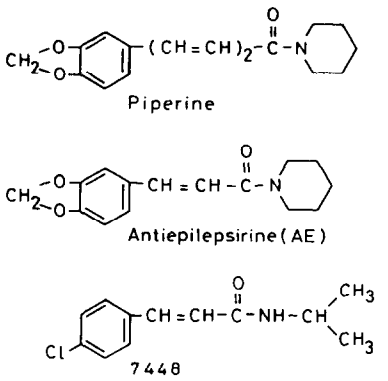

Fig. 1. Chemical structures of piperine, AE and 7448.

MATERIALS AND METHODS

Male CD–COBS rats (Charles River, Italy) weighing 200 ± 20 g were used in these experiments. Animals were maintained at constant room temperature (21 ± 1°) and relative humidity (60%) and a 12 hr light–dark cycle with free access to water and food. Rats were killed by decapitation. For biochemical determinations striata and limbic area containing the nucleus accumbens, olfactory, tuberculum and amygdaloid nucleus were dissected and frozen on dry ice.

Blood was collected from the decapitated animals, and serum was separated by centrifuging the blood after clotting. Brain and serum samples were kept at −80° until biochemical assay.

For *in vitro* studies whole brain without cerebellum or cerebral cortex, limbic area and hippocampus was used for ^{3}H-5HT release and ^{3}H-5HT binding studies. Pooled areas were homogenized immediately after dissection. Some of the compounds tested were generously donated by Prof. Pei (Beijing Medical College, People's Republic of China). All the compounds were suspended in 2% Tween-80 and administered intraperitoneally. Control groups were treated with vehicle. A dose of 150 mg/kg was chosen since this has generally been used for pharmacological investigations [3].

Biochemical determinations

5-Hydroxytryptamine (5HT), its metabolite 5-hydroxyindoleacetic acid (5HIAA), and its precursor

* Present address: Department of Pharmacology, Nanking College of Pharmacy, Nanking, People's Republic of China.

† On leave of absence from Department of Pharmacology, School of Dentistry, Iwate Medical University, 020 Morioka, Japan.

tryptophan (TP) were measured by high pressure liquid chromatography coupled with electrochemical detection as described before [8, 9].

Free TP was separated from albumin-bound TP by centrifuging 1 ml serum in dialysis tubing (size 8/32 Visking Co.) at 3000 *g* for 2 hr at 0–4° [10]. TP was determined in the dialyzate (free TP) and in whole serum (total TP).

In vitro *studies*

^{3}H-5HT binding. The method was essentially the same as described by Bennett and Snyder [11]. The binding assays consisted of 1 ml of 0.05 M Tris–HCl buffer pH 7.4, containing 4 mM $CaCl_2$, 10 μM pargyline, 0.1% ascorbic acid and 2 nM ^{3}H 5HT (final concentrations) to which 1 ml of membrane preparation (corresponding to 20 mg of original wet weight) was added. After 15 min incubation at 37° samples were rapidly vacuum filtered. Non-specific binding was determined in the presence of 10 μM unlabelled 5HT, and was about 40% of total ^{3}H-5HT binding.

^{3}H-5HT release. Synaptosomes were obtained as described by Mennini *et al.* [12]. 5HT release was studied by a superfusion technique as reported by Mennini *et al.* [13]. Pooled synaptosome suspensions were preincubated for 15 min at 37° in the presence of 0.1 μM ^{3}H-5HT (12 Ci/mmole, Radiochemical Int., Amersham). One ml portions of the suspension were filtered through Millipore filters, washed and placed on the bottom of a superfusion chamber [14]. The drug was dissolved in Krebs–Henseleit solution and superfused from time 0 to 20 min at a constant rate of 0.5 ml/min. The effluent was collected directly into liquid scintillation vials every 5 min. The radioactivity remaining on the filters at the end of superfusion was also counted, and was used for calculation of the percentage of total radioactivity released (i.e. total fractions recovered plus filter).

Statistical analysis

All data were analyzed by analysis of variance and statistical significance was determined by Dunnett's test.

RESULTS

The three anticonvulsants tested, AE, piperine and compound 7478, caused a marked increase in 5HIAA without changing the levels of 5HT (Table 1) 60 min after administration, a time interval in which the substance showed anticonvulsant activity. Compound 7448 appeared somewhat less effective than piperine and AE.

The effect of AE on the serotonergic system was long-lasting, as seen in two separate experiments (Table 2), in which the effects on 5HT and 5HIAA were studied in limbic and striatal areas at longer

Table 1. Effect of AE and its derivatives on 5HT and 5HIAA concentrations in rat limbic area

	Dose (mg/kg i.p.)	Time (min)	Concentrations in limbic area (ng/g) 5HT	5HIAA
Control	—	0	690 ± 43	450 ± 35
Piperine	150	60	683 ± 55	806 ± 48*
AE	150	60	659 ± 20	761 ± 79*
7448	150	60	579 ± 43	574 ± 33†

Each value is the mean ± S.E. of 6 animals.
* $P < 0.01$ compared to controls.
† $P < 0.05$ compared to controls.

Table 2. Effect of AE on TP, 5HT and 5HIAA concentrations in the rat limbic area and striatum

Exp. No.	Time (hr) after AE (')	Limbic area TP	5HT	5HIAA	Striatum TP	5HT	5HIAA
	0	4020 ± 310	515 ± 20	490 ± 35	3700 ± 280	260 ± 19	338 ± 24
	0.5	n.d.	562 ± 30	567 ± 38	n.d.	226 ± 22	306 ± 7
I	1	7490 ± 350*	545 ± 38	685 ± 40†	6320 ± 280*	272 ± 23	363 ± 19
	2	7850 ± 470*	522 ± 22	751 ± 28†	7130 ± 310*	214 ± 15	412 ± 17*
	4	6880 ± 380*	758 ± 57*	871 ± 74*	5680 ± 210*	302 ± 21	545 ± 7*
	8	6100 ± 350*	582 ± 8†	925 ± 61*	4960 ± 270*	310 ± 16	891 ± 55*
	0	3680 ± 186	493 ± 20	496 ± 30	3920 ± 218	248 ± 20	330 ± 26
	3	4254 ± 150†	598 ± 33	768 ± 68†	4846 ± 250†	269 ± 23	424 ± 20†
II	6	5740 ± 622*	769 ± 62*	1160 ± 150*	5818 ± 415*	355 ± 38	482 ± 25*
	12	6790 ± 415*	741 ± 96†	1024 ± 126*	7040 ± 380*	387 ± 41*	629 ± 50*
	24	5050 ± 433	631 ± 41	750 ± 56†	5980 ± 650	281 ± 16	540 ± 47*

(') AE was injected i.p. (150 mg/kg).
Data are expressed as ng/g and each value is the mean ± S.E. of 6 samples.
Different from control (time 0): * $P < 0.01$ by Dunnett's test; † $P < 0.05$ by Dunnett's test.

Table 3. Total and free tryptophan in plasma of rats treated with AE or Na salycilate

	Total TP	Free TP	Free TP/Total TP
Control	23.3 ± 1.1	1.7 ± 0.1	0.07
AE	13.1 ± 0.9*	4.6 ± 0.2*	0.35
Na salicylate	12.2 ± 0.5	7.0 ± 0.4*	0.59

The results are expressed as μg/ml and are the means ± S.E. of 6 samples.
AE: 150 μg/kg i.p. Na salicylate: 300 mg/kg i.p.
Drugs were given 2 hr before killing.
* Different from control $P < 0.01$ by Dunnett's test.

intervals after drug administration. The rise in 5HIAA lasted at least 12 hr in limbic area and up to 24 hr in striatum. At longer intervals AE also raised 5HT, though not to the same extent and not as uniformly as 5HIAA. In fact, while 5HIAA levels almost doubled, 5HT showed only a 50% increase.

Table 2 also gives the levels of TP, the 5HT precursor, in brain tissues. This aminoacid was elevated by AE treatment and its concentration was still high 12 hr after treatment.

The drug also raised the plasma concentration of free TP. In fact, as shown in Table 3, similarly to Na salicylate, a drug whose action on albumin-bound TP is well documented [10, 15–17] AE raised the ratio of free-to-total TP by reducing total and increasing free tryptophan. The data presented in Fig. 2 indicates that AE was effective in releasing ^{3}H-5HT from an *in vitro* synaptosomal preparation, in a concentration-dependent manner starting from 10^{-6} M. In contrast, AE up to the concentration of 10^{-4} M had virtually no effect in displacing ^{3}H-5HT from high-affinity brain binding sites *in vitro* in membrane preparations from rat cerebral cortex, limbic area and hippocampus (data not shown).

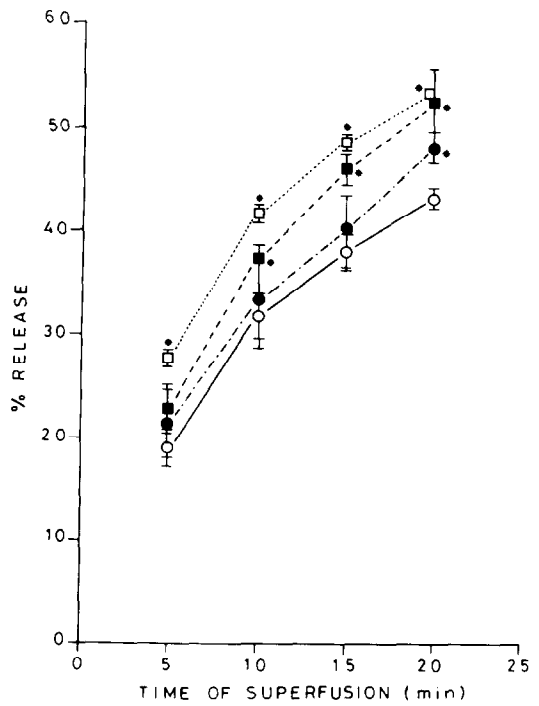

Fig. 2. Effect of AE on synaptosomal ^{3}H-5HT release. Percentage release of ^{3}H-5HT is plotted vs time (min) in a superfused system which avoids reuptake. The release at each time is expressed as cumulative percentage of total radioactivity recovered (see Materials and Methods for details). Each point is the mean ± S.D. of 3 replications. (—○—) control; (—●—) AE 10^{-6} M; (-■-) AE 10^{-5} M; (···□···) AE 10^{-4} M. *$P < 0.01$, Dunnett's test.

DISCUSSION

Piperine derivatives, particularly AE, were very active in our conditions in raising the levels of 5HIAA in rat brain striatum and limbic area. This effect is probably due to an enhancement of synthesis and release of 5HT as suggested by the fact that AE enhanced 5HT release from a synaptosomal preparation and by the finding that the levels of aminoacid precursor and, sometimes, of the neurotransmitter itself were raised to some extent. An increase in brain TP is currently considered a stimulus for 5HT synthesis [16–18] since brain tryptophan hydroxylase is not saturated by the physiological concentration of brain TP [19].

The elevation of TP in the brain was probably caused by the action of AE on circulating TP. In fact, similarly to Na salicylate, a drug known to displace albumin-bound TP [10, 15, 17], AE increased the ratio of free-to-total TP (Table 3). The effect on 5HT synthesis through raising the concentration of precursor is not, however, the only action of AE on brain 5HT. Our *in vitro* results (Fig. 2) showed a clear releasing effect from synaptosomes, suggesting that AE may also release 5HT from nerve endings.

On the other hand, the observed increase in 5HT metabolism is probably unrelated to a positive feedback mechanism caused by 5HT receptor blockade [20], because AE was unable to displace 5HT from specific binding sites. However, the hypothesis of the formation of an AE metabolite with antagonistic properties cannot be completely excluded, especially in view of the relatively slow onset of AE's effect and its very long duration of action.

We tend instead to exclude an inhibitory effect on active transport of the monoamine acid metabolites similar to that of probenecid [21]. In fact when present, this action is evident also on the DA metabolites homovanillic acid (HVA) and hydroxyphenyl-

acetic acid (DOPAC) [22]. In contrast, in some preliminary experiments (data not shown in this paper) we observed that AE and Na salicylate raised the concentration of HVA, suggesting that they affect the metabolism of dopamine, but did not change DOPAC concentration, thus excluding any action on the transport of acid monoamine metabolites.

Whether AE and other piperine derivatives exert their anticonvulsant activity because of this effect on the synthesis of brain 5HT cannot be discussed at the present time. There are previous papers supporting this view [5, 23, 24], but more recent work from another laboratory tends to exclude the involvement of 5HT in convulsive conditions [25–27].

In the light of AE's antagonistic action on picrotoxine-induced convulsions, and the well-known anti-GABA activity of picrotoxine, the possible effects of AE on the GABAergic system should be considered in future research on the piperine derivatives.

Acknowledgements—This work was supported by Contract CNR "Gruppo Nazionale di Farmacologia" No. Ct. 82.01300.04 and by a fellowship to G. Q. Liu from WHO. The authors would like to express their appreciation to Prof. Y. A. Pei in Beijing Medical College for the gift of compounds and for beneficial discussion, also to Mr. S. Bernasconi and G. Rinaldi for their technical assistance.

REFERENCES

1. Y. Pei, J. S. Li, Z. J. Cai, B. H. Zhang, C. Tao and B. S. Ku, *Chinese J. Med.* **58**, 216 (1978).
2. A. Balsamo, P. L. Barili, P. Crotti, B. Macchia, F. Macchia, A. Cuttica and N. Passerini, *J. med. Chem.* **20**, 48 (1977).
3. Y. Pei, W. Yue, J. R. Cui and H. Y. Yao, *Acta pharmac. Sinica* **15**, 198 (1980).
4. Z. S. Zhou, *J. Beijing Med. College* **13**, 1 (1981).
5. W. D. Gray and C. E. Rauh, *J. Pharmac. exp. Ther.* **177**, 206 (1971).
6. M. Kilian and H.-H. Frey, *Neuropharmacology* **12**, 681 (1973).
7. G. R. Wenger, R. E. Stitzel and C. R. Craig, *Neuropharmacology* **12**, 693 (1973).
8. F. Ponzio and G. Jonsson, *J. Neurochem.* **32**, 129 (1979).
9. D. D. Koch and P. T. Kissinger, *J. Chromatogr.* **164**, 441 (1979).
10. H. Iwata, H. Okamoto and S. Koh, *Jap. J. Pharmac.* **25**, 303 (1975).
11. J. P. Bennett Jr. and S. H. Snyder, *Molec. Pharmac.* **12**, 373 (1976).
12. T. Mennini, R. Pataccini and R. Samanin, *Br. J. Pharmac.* **64**, 75 (1978).
13. T. Mennini, E. Borroni, R. Samanin and S. Garattini, *Neurochem. Int.* **3**, 289 (1981).
14. M. Raiteri, F. Angelini and G. Levi, *Eur. J. Pharmac.* **25**, 411 (1974).
15. G. Curzon and A. R. Green, *Br. J. Pharmac.* **39**, 653 (1970).
16. D. G. Grahame-Smith, *J. Neurochem.* **18**, 1053 (1971).
17. A. Tagliamonte, P. Tagliamonte, J. Perez-Cruet, S. Stern and G. L. Gessa, *J. Pharmac. exp. Ther.* **177**, 475 (1971).
18. G. L. Gessa, G. Biggio and A. Tagliamonte, *Fedn Proc. Fedn Am. Socs exp. Biol.* **31**, abstract 2168, 599 (1972).
19. D. A. V. Peters, P. L. McGeer and E. G. McGeer, *J. Neurochem.* **15**, 1431 (1968).
20. R. Invernizzi and R. Samanin, *Pharmac. Res. Commun.* **13**, 511 (1981).
21. D. F. Sharman, *Br. J. Pharmac. Chemother.* **30**, 620 (1967).
22. P. F. Spano and N. H. Neff, *Brain Res.* **42**, 139 (1972).
23. P. C. Jobe, A. L. Pichioni and L. Chin, *Life Sci.* **13**, 1 (1973).
24. H. Meyer and H.-H. Frey, *Neuropharmacology* **12**, 939 (1973).
25. A. Quattrone, V. Crunelli and R. Samanin, *Neuropharmacology* **17**, 643 (1978).
26. V. Crunelli, S. Bernasconi and R. Samanin, *Psychopharmacology* **66**, 79 (1979).
27. V. Crunelli, L. Cervo and R. Saminin, in *Neurotransmitters, Seizures and Epilepsy* (Eds. P. L. Morselli, K. L. Lloyd, W. Löscher, B. Meldrum, B. Chir, and E. H. Reynolds), p. 195. Raven Press, New York (1981).