Biochemical Pharmacology, 1966, Vol. 15, pp. 703–710. Pergamon Press Ltd., Printed in Great Britain.

INHIBITION OF Na^+K^+-ACTIVATED ADENOSINE TRIPHOSPHATASE ACTIVITY IN RAT BRAIN BY SUBSTITUTED PHENOTHIAZINES*

P. W. DAVIS and T. M. BRODY

Department of Pharmacology,
University of Michigan Medical School, Ann Arbor, Mich., U.S.A.

(*Received* 15 *November* 1965; *accepted* 22 *December* 1965)

Abstract—The effects of fifteen substituted phenothiazines and certain other drugs were studied on microsomal Na^+K^+-activated ATPase in rat brain to attempt to relate their central nervous system effects with their inhibition of enzyme activity. The mean control activity of the preparation was $14{\cdot}4 \pm 0{\cdot}2$ μmoles P_i/mg protein/15 min at 37° in the presence of Na^+, K^+, and Mg^{++}; and $3{\cdot}3 \pm 0{\cdot}1$ in the presence of Mg^{++} alone.

Certain substitutions on the phenothiazine nucleus were noted to increase the inhibitory potency. At position 2 of the phenothiazine nucleus, a trifluoromethyl group was more potent than a chloro group which was in turn more potent than hydrogen. At position 10, a piperazine side chain was more inhibitory than an aliphatic amine substitution. The sulfoxide derivatives of promazine, chlorpromazine, and triflupromazine did not inhibit Na^+K^+-ATPase activity at concentrations as high as 5×10^{-4} M.

Compounds which failed to influence Na^+K^+-ATPase activity at concentrations used in the phenothiazine studies were pentylenetetrazol, *d*-amphetamine, strychnine, γ-aminobutyric acid, and pentobarbital.

The evidence in this report suggests that, for substituted phenothiazines, there may be a correlation between inhibition of brain Na^+K^+-ATPase activity *in vitro* and certain central nervous system effects *in vivo*.

A Na^+K^+-activated Mg^{++}-dependent adenosine triphosphatase was first reported in crab nerve by Skou in 1957.[1] Subsequent work, summarized in recent reviews, has established the nearly ubiquitous occurrence of a similar Na^+K^+-activated ATPase in most of the biological tissues that have been studied and has also established a striking correlation between the properties of this enzymatic system and features of active transport of monovalent cations.[2, 3]

Inhibition of Na^+K^+-activated ATPase activity in rat brain by phenothiazines such as promethazine and chlorpromazine has been reported previously.[4, 5]

The purpose of this report is to present recent findings which suggest a correlation between the potency of phenothiazines inhibiting brain Na^+K^+-ATPase *in vitro* and the tranquilizing potency of the same agents *in vivo*.

EXPERIMENTAL

Male albino rats (250–350 g) were sacrificed by decapitation. The entire cerebrum, cerebellum, and brain stem were removed from each of four animals and pooled

* A preliminary account of this work was presented before the American Society for Pharmacology and Experimental Therapeutics on August 17, 1965, in Philadelphia, Pa. (*Pharmacologist* **7**, 141, 1965).

immediately in ice-cold 0·25 M sucrose. All subsequent operations were performed at temperatures of 0–4°. A 1:10 tissue homogenate in 0·25 M sucrose was prepared in a hand-driven Dounce ball-type homogenizer. Unbroken cells, nuclei, and mitochondria were removed by centrifugation at 1000 *g* for 20 min and 12,000 *g* for 30 min in a Serval RC2 centrifuge. The 12,000-*g*/30-minutes supernatant was placed in a Spinco model L ultracentrifuge at 35,000 *g* for 30 min. After removal of the 35,000-*g* supernatant by decantation, the heavy microsomal pellet was gently resuspended by hand homogenization in 0·25 M sucrose and subjected to a second 35,000-*g* centrifugation for 30 min. The heavy microsomal pellet was then resuspended in 0·25 M sucrose and diluted with an equal volume of 0·2% deoxycholic acid after neutralization to pH 7·5 with 0 1 M Tris. The preparation was stored at 0° for 2 to 3 days prior to use. The use of deoxycholic acid increased total activity (Na,K,Mg) slightly while increasing the NaKMg/Mg activity ratio with a twofold increase in the NaK component. This is in confirmation of the findings of others who have used the brain microsomal preparation.[6–8]

The Na^+K^+-ATPase activity of the heavy microsomal suspension was measured under the following conditions: Tris-ATP (Sigma Chemical Co.) 3 mM; $MgCl_2$ 3 mM; NaCl 100 mM; KCl 15 mM; choline Cl 115 mM (as an osmotic substitute in tubes where Na^+ and K^+ were omitted); Tris-HCl 30 mM at pH 7·5; and 0·2 ml of the heavy microsomal suspension in a total incubation volume of 2·0 ml. Incubation was at 37° for 15 min and was terminated by the addition of 1·0 ml of 1·5 N perchloric acid. After removal of the precipitated protein by centrifugation, an aliquot of the incubation medium was assayed for inorganic phosphate by the Fiske and SubbaRow method.[9] The protein content of the preparation was determined by a modified biuret technique.[10]

Phenothiazines tested as inhibitors of brain Na^+K^+-ATPase activity included: promazine HCl, promethazine HCl, and propiomazine HCl (Wyeth Laboratories); chlorpromazine HCl, prochlorperazine edisylate, and trifluoperazine diHCl (Smith, Kline & French Laboratories); thioridazine HCl and thiethylperazine maleate (Sandoz Pharmaceuticals); mepazine HCl (Warner-Chilcott Laboratories); perphenzine (Schering Corp.); triflupromazine (Squibb & Sons); and fluphenazine diHCl (White Laboratories). The phenothiazine analog imipramine (Geigy Pharmaceuticals) was employed also. The sulfoxide derivatives of promazine, chlorpromazine, and trifluoperazine were kindly supplied by Dr. Harry Green, Smith, Kline & French Laboratories.

RESULTS

Control ATPase activity in the presence of Na^+, K^+, and Mg^{++} was 14·4 $\pm$ 0·2 μmoles P_i/mg protein/15 min at 37° as compared with 3·3 $\pm$ 0·1 obtained in the presence of Mg^{++} alone; i.e. a fourfold increase above Mg^{++}-dependent ATPase activity as a result of Na^+K^+-activation. In all experiments, drugs to be tested were included in the Mg as well as in the NaKMg tubes. All data are expressed as per cent inhibition of Na^+K^+-ATPase activity of the drug-treated enzyme compared with control—Na^+K^+ATPase activity being defined as the difference between the total activity (NaKMg) and Mg^{++}-dependent ATPase activity.

TABLE 1. INHIBITION OF Na^+K^+-ATPase ACTIVITY BY PHENOTHIAZINES

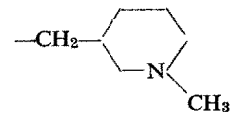
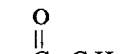
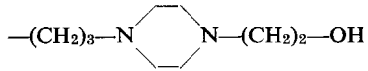
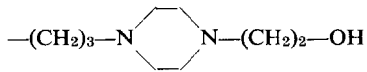
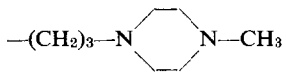
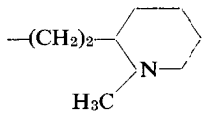
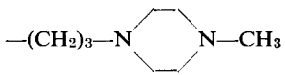
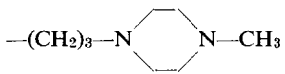
Compound N	R_2	R_1	% Inhibition of Na^+K^+-ATPase* 1×10^{-4}M	5×10^{-5}M
1. Promazine sulfoxide (3) sulfoxide of compound 4 below			1·0 ± 1·0	−1·3 ± 3·2
2. Chlorpromazine sulfoxide (3) sulfoxide of compound 8 below			3·7 ± 3·1	2·0 ± 2·5
3. Trifluoperazine sulfoxide (3) sulfoxide of compound 16 below			2·7 ± 2·7	0·0 ± 4·0
4. Promazine (5)	H	$-(CH_2)_3-N(CH_3)_2$	6·4 ± 2·0	1·0 ± 2·1
5. Imipramine (3)†	H	$-(CH_2)_3-N(CH_3)_2$	9·3 ± 2·9	6·7 ± 2·6
6. Promethazine (5)	H	$-CH_2-CH(CH_3)-N(CH_3)_2$	30 ± 3·0	15 ± 3·0
7. Mepazine (5)	H		29 ± 1·4	14 ± 4·0
8. Chlorpromazine (5)	Cl	$-(CH_2)_3-N(CH_3)_2$	38 ± 2·4	17 ± 2·4
9. Propiomazine (3)		$-CH_2-CH(CH_3)-N(CH_3)_2$	35 ± 4·0	18 ± 1·4

TABLE 1.—*continued*

Compound N	R_2	R_1	% Inhibition of Na^+K^+-ATPase* 1×10^{-4}M	5×10^{-5}M
10. Perphenazine (3)	Cl		55 ± 3·7	19 ± 2·6
11. Trifluprom azine (5)	CF_3	$-(CH_2)_3-N(CH_3)_2$	65 ± 2·3	23 ± 2·7
12. Fluphenazine (5)	CF_3		80 ± 0·9	30 ± 2·1
13. Prochlorperazine (3)	Cl		88 ± 1·2	33 ± 3·2
14. Thioridazine (3)	$-S-CH_3$		86 ± 1·9	45 ± 2·7
15. Thiethylperazine (3)	$-S-C_2H_5$		91 ± 2·6	41 ± 2·3
16. Trifluoperazine (3)	CF_3		98 ± 1·3	50 ± 4·4

* Mean ± S.E.
† Nonphenothiazine analog of promazine.

Values for inhibition of Na^+K^+-ATPase activity by various substituted phenothiazines are presented in Table 1. Each value listed is the mean of either three or five independent experiments as indicated. Each individual experiment within such a series was performed on a different day, in duplicate, with microsomal suspensions from different animals and independently prepared drug solutions. The per cent

STATISTICAL COMPARISONS FROM TABLE 1 DATA

	P value	
	1×10^{-4}M	5×10^{-5}M
Trifluprομazine vs. chlorpromazine	<0·01	NS*
Chlorpromazine vs. promazine	<0·001	<0·01
Trifluoperazine vs. prochlorperazine	<0·02	<0·05
Fluphenazine vs. perphenazine	<0·005	NS
Prochlorperazine vs. chlorpromazine	<0·001	<0·05
Trifluoperazine vs. trifluprομazine	<0·005	<0·005
Fluphenazine vs. trifluprομazine	<0·05	NS
Promazine vs. promazine sulfoxide†	NS	NS
Chlorpromazine vs. chlorpromazine sulfoxide	<0·001	<0·025
Trifluoperazine vs. trifluoperazine sulfoxide	<0·001	<0·005

* NS = not significant at 0·05 level.

† At a higher concentration of the drugs (5×10^{-4}M), where the inhibition values for promazine and promazine sulfoxide were 77·0 ± 1·8 (N = 5) and −1·0 ± 4·2 (N = 3), respectively, the difference was significant, $P < 0·001$.

inhibition values in a single experiment were calculated on the basis of the control activity of that day.

The substituted phenothiazines studied differed from one another in the R_1 and R_2 substituents at positions 10 and 2 of the phenothiazine nucleus (Fig. 1). Two patterns

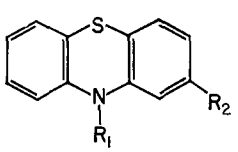


FIG. 1. Substituted groups on the phenothiazine nucleus.

of structure–activity relationships were noted between substituted groups on the phenothiazine nucleus and inhibition of Na^+K^+-ATPase.

Certain changes in the R_2 group resulted in marked differences in inhibitory potency. A trifluoromethyl substitution inhibited more than a corresponding chloro group which, in turn, was more potent than hydrogen substituted in the same position. For example, 1×10^{-4} M trifluprομazine (which contains a trifluoromethyl group at R_2) inhibited enzyme activity by 65 per cent as compared to only 38 per cent inhibition obtained with the chloro analog, chlorpromazine, at the same concentration. Chlorpromazine at 1×10^{-4} M was more inhibitory than promazine which has an unsubstituted hydrogen at R_2 and which inhibited only 6 per cent at this concentration. The inhibition by promazine at 10^{-4} M did not differ significantly from control activity at the 5 per cent confidence level. However, a higher concentration of this compound (5×10^{-4} M) reduced control activity by 77 ± 1·8% (N = 5). The values for other agents at this concentration are not presented in Table 1 since all compounds

gave complete inhibition of enzyme activity. Triflupromazine, chlorpromazine, and promazine differ in structure only at R_2, the R_1 group being dimethylaminopropyl, common to all three compounds.

Another example of the increased potency of a trifluoromethyl substitution relative to a chloro group was noted in the 50 per cent inhibition by trifluoperazine as compared with an inhibition of only 33 per cent by prochlorperazine when 5×10^{-5} M of each agent was compared with control activity. These two compounds have identical piperazine side-chain moieties and differ structurally only at R_2. A third illustration of the greater inhibitory potency of a trifluoromethyl group was seen with fluphenazine and perphenazine. Fluphenazine with a trifluoromethyl group at R_2 inhibited 80 per cent at 1×10^{-4} M, whereas the chloro analog, perphenazine, inhibited only 55 per cent at the same concentration.

In addition to the above structural changes in the R_2 group, the nature of the substituent in R_1 also appeared to be a factor in determining inhibitory potency. In general, a side chain containing a piperazine moiety led to greater reduction in control activity than did an aliphatic amine side chain. In comparing prochlorperazine and chlorpromazine, differing only in the R_1 moiety, the former, which has a piperazine side chain, inhibited 88 per cent at 1×10^{-4} M, whereas the latter, an aliphatic amine, produced a fall of only 38 per cent in enzyme activity at the same concentration. Also, the piperazine compounds trifluoperazine and fluphenazine were more potent at 1×10^{-4} M (inhibiting 98 per cent and 80 per cent respectively) than the aliphatic amine triflupromazine which reduced enzyme activity by only 65 per cent. These three compounds differ only at R_1, since R_2 is trifluoromethyl in each. In addition, although no direct comparison was possible since the R_2 groups are not constant, the aliphatic amines promethazine and propiomazine produced only moderate inhibition when compared to the more potent piperazine compounds.

Promazine sulfoxide, chlorpromazine sulfoxide, and trifluoperazine sulfoxide did not significantly inhibit Na^+K^+-ATPase activity at 1×10^{-4} M, whereas the parent compounds, promazine, chlorpromazine, and trifluoperazine, produced 6, 38 and 98 per cent inhibition respectively at the same concentration.

Imipramine, a nonphenothiazine analog of promazine, produced weak inhibition quite similar in magnitude to that seen with promazine. While imipramine did not significantly reduce Na^+K^+-ATPase activity at 1×10^{-4} M, a higher concentration of this compound (5×10^{-4} M) produced $59 \pm 6{\cdot}4$ per cent inhibition (mean $\pm$ S.E., $N = 3$), a value similar to that obtained with promazine at the same concentration. With regard to piperidyl structures, mepazine produced moderate inhibition, whereas marked inhibition was obtained with thioridazine.

Compounds that failed to influence Na^+K^+-ATPase activity at the concentrations used in the phenothiazine studies included pentylenetetrazol, *d*-amphetamine, strychnine, γ-aminobutyric acid, and pentobarbital. These compounds were ineffective in concentrations as high as 5×10^{-4} M.

DISCUSSION

Substituted phenothiazines exert numerous effects upon the central nervous system. Commonly measured parameters include antiemetic action, extrapyramidal signs, depression of conditioned-avoidance responses, and antipsychotic effects clinically. In attempting to correlate enzyme inhibition *in vitro* with central nervous

system actions *in vivo*, any of these central nervous system actions might conceivably be selected as a response correlate *in vivo*.

It is interesting to note that the structure–activity relationships between substituted phenothiazines and inhibition *in vitro* of Na^+K^+-ATPase reported in this paper are in accord with several major structure–activity relationships generally considered in regard to certain effects of substituted phenothiazines *in vivo* on the central nervous system. The only compound that appears to fall out of line is thioridazine which, on clinical impression and other studies *in vivo*, has a potency comparable to or possibly even weaker than chlorpromazine, yet in the present study appears to be as potent an inhibitor as prochlorperazine.

Increased potency for depression of conditioned-avoidance responses in animals and increased antipsychotic action clinically in humans has resulted from substitutions in the R_2 group where a trifluoromethyl moiety is more potent than a chlorine substitution which in turn is more potent than hydrogen; e.g. trifluprομazine > chlorpromazine > promazine. Another example is the greater potency of trifluoperazine relative to that of prochlorperazine.[11]

These same central nervous system actions have been shown to be greater for piperazine structures than for aliphatic amines in the R_1 group; e.g. prochlorperazine > chlorpromazine. Also, both trifluoperazine and fluphenazine exert stronger actions than trifluprομazine. The sulfoxide metabolites which fail to depress Na^+K^+-ATPase in the present study are known to possess little central nervous system activity.[12, 13]

In recent years many studies have suggested correlations between various biochemical and physicochemical effects of the substituted phenothiazines and their clinical effectiveness as tranquilizers. Much of this work has been recently reviewed by Guth and Spirtes,[14] who offer a unifying hypothesis suggesting that changes in membrane permeability induced by phenothiazines may be the basis for many of the reported biochemical actions of phenothiazines. They postulate that the phenothiazines act as "membrane stabilizers". In view of the present report of phenothiazine inhibition of brain Na^+K^+-ATPase, an enzyme believed to be intimately involved in active transport of monovalent cations across membrane barriers, it is tempting to speculate that inhibition of such an enzyme might explain the changes in ion and water transfer processes seen in studies of changes in membrane permeability. Thus, these data are not inconsistent with the hypothesis that the phenothiazines act by membrane stabilization and suggest in addition an enzyme site where such an effect may occur.

Two other classes of pharmacological agents are also capable of inhibiting Na^+K^+-ATPase activity—the cardiac glycosides[15, 16] and the organic mercurial diuretics.[17, 18] Repke[19] has correlated the inhibition of Na^+K^+-ATPase with the chemical structure and potency of the various cardiac glycosides, and Jones *et al.*[20] have attempted to relate diuretic potency and ATPase inhibition of certain diuretics. It is interesting to note that the phenothiazine tranquilizers can produce ventricular fibrillation in man when large doses of the drug are administered,[21] an action characteristic of cardiac glycoside toxicity.

The question of whether the drug concentrations used in the present study have any relevance to those one might expect *in vivo*, is difficult to answer because of the nature of the compounds. Generally the concentrations used here are of the same order of

magnitude as those used in other experiments *in vitro*.[14] Several studies have demonstrated that the phenothiazines are avidly taken up by tissues, particularly brain. For example, Saltzman and Brodie[22] found chlorpromazine to be concentrated some 80-fold in brain after administration to dogs. De Jaramillo and Guth[23] have determined that the levels of phenothiazines in the hypothalamus of the dog might be as high as 5×10^{-4} M, with possibly still higher concentrations in subcellular components.[24, 25] On the other hand, Gillette[26] has postulated that because the percentage of binding of the phenothiazines is so high and because the binding is reversible, the amount of free or unbound drug must be extremely small (about 5×10^{-7} M). He suggests that concentrations in this range are those that should be considered to interact with the receptor to produce the pharmacological effect. Since no measurements of free drug were made in the present study, it is impossible to assess this aspect of the work. However, none of the above considerations negates the findings of a positive correlation between potency *in vivo* and ability of these agents to inhibit brain Na^+K^+-ATPase activity.

REFERENCES

1. J. C. SKOU, *Biochim. biophys. Acta.* **23**, 394 (1957).
2. J. D. JUDAH and K. AHMED, *Biol. Rev.* **39**, 160 (1964).
3. J. C. SKOU, *Physiol. Rev.* **45**, 596 (1965).
4. J. D. JUDAH and K. AHMED, *J. cell. comp. Physiol.* **64**, 355 (1964).
5. J. JÄRNEFELT, *Biochim. biophys. Acta* **59**, 643 (1962).
6. J. C. SKOU, *Biochim. biophys. Acta* **58**, 314 (1962).
7. J. JÄRNEFELT, *Biochem. biophys. Res. Commun.* **17**, 330 (1964).
8. R. W. ALBERS, G. RODRIGUEZ and E. DE ROBERTIS, *Proc. nat. Acad. Sci., Wash.* **53**, 557 (1965).
9. C. H. FISKE and Y. SUBBAROW, *J. biol. Chem.* **66**, 375 (1925).
10. A. G. CORNWALL, C. J. BARDAWILL and M. M. DAVID, *J. biol. Chem.* **177**, 751 (1949).
11. E. F. DOMINO, *Clin. Pharmac. Ther.* **3**, 599 (1962).
12. N. C. MORAN and W. M. BUTLER, JR., *J. Pharmac. exp. Ther.* **118**, 328 (1956).
13. J. D. DAVIDSON, L. L. TERRY and A. SOJERDSMA, *J. Pharmac. exp. Ther.* **121**, 8 (1957).
14. P. S. GUTH and M. A. SPIRTES, *Int. Rev. Neurobiol.* **7**, 231 (1964).
15. R. L. POST, C. R. MERRIT, C. R. KINSOLVING and C. D. ALBRIGHT, *J. biol. Chem.* **235**, 1796 (1960).
16. E. T. DUNHAM and I. M. GLYNN, *J. Physiol., Lond.* **156**, 274 (1961).
17. E. J. LANDON and R. L. NORRIS, *Biochim. biophys. Acta* **71**, 266 (1963).
18. C. B. TAYLOR, *Biochem. Pharmac.* **12**, 539 (1963).
19. K. REPKE, in *Drugs and Enzymes*, Proc. 2nd Int. Pharmacological Meeting, Prague, 1963.
20. V. D. JONES, G. LOCKETT and E. J. LANDON, *J. Pharmac. exp. Ther.* **147**, 23 (1965).
21. L. E. HOLLISTER and J. C. KOSEK, *J. Am. med. Ass.* **192**, 1035 (1965).
22. N. P. SALTZMAN and B. B. BRODIE, *J. Pharmac. exp. Ther.* **118**, 46 (1956).
23. G. A. V. DE JARAMILLO and P. S. GUTH, *Biochem. Pharmac.* **12**, 525 (1963).
24. P. S. GUTH and J. AMARO, *Biochem. Pharmac.* **14**, 67 (1965).
25. J. B. RAGLAND, *Psychopharmacol. Serv. Cent. Bull.* **2**(5), 80 (1962).
26. J. R. GILLETTE, In *Drugs and Enzymes*, Proc. 2nd Int. Pharmacological Meeting, Prague (1963).