Biochemical Pharmacology, Vol. 29, pp. 2413–2416.

0006-2952/80/0915-2413 $02.00/0

INHIBITION OF HIGH AFFINITY CHOLINE UPTAKE

STRUCTURE ACTIVITY STUDIES

FREDERICK BATZOLD*, ROBERT DEHAVEN†, MICHAEL J. KUHAR†,‡ and NIGEL BIRDSALL§

Departments of †Pharmacology and Experimental Therapeutics and Psychiatry and Behavioral Sciences, Johns Hopkins University, School of Medicine, Baltimore, MD 21205, U.S.A.; *Department of Pharmacology and Experimental Therapeutics, The Albany Medical College of Union University, Albany, NY 12208, U.S.A.; and §National Institute for Medical Research, London, U.K.

(*Received* 19 *November* 1979; *accepted* 11 *February* 1980)

Abstract—Sodium-dependent, high affinity choline uptake in brain synaptosomes was inhibited by various choline analogues. When substituents were placed on the quaternary nitrogen, the compounds became weaker inhibitors as the bulk of the substituents increased. None of the compounds with changes in the hydroxyl group part, except for a bis-quaternary compound, were potent inhibitors. Increasing the oxygen–nitrogen distance resulted in a weaker inhibition. Data from crystallographic studies [M. E. Senko and M. Templeton, *Acta crystallogr.* **13**, 281 (1960); and F. G. Canepa, *Nature, Lond.* **207**, 1152 (1965)] and inspection of Dreiding molecular models suggest an optimal oxygen–nitrogen distance of about 3.3 Å. These results support earlier suggestions that a hydroxyl group and a quaternary nitrogen are necessary for interaction with the carrier.

The high affinity choline transport system in cholinergic neurons has many properties suggesting that it is a rate-limiting and regulatory step in the synthesis of acetylcholine (for review, see Ref. 1). While there have been suggestions and speculations, the mechanism of the transport system is unknown. One can get some information about the active site of the carrier by structure–activity studies. We and others have previously studied the inhibition of choline uptake by various choline analogues [2–4]. Other workers have also examined the uptake, acetylation and release of various choline analogues [5–10]. Taken together, these studies suggest certain structural requirements for transport by the high affinity carrier. It appears that a free hydroxyl group is necessary (however, bis-quaternary compounds without a hydroxyl are good inhibitors of transport; see below). Also, it appears that a quaternary nitrogen group is necessary; however, the methyl groups can be replaced by ethyl groups which suggests that only limited bulk on the nitrogen is acceptable. There is some evidence that the hydroxyl-quaternary nitrogen distance can be increased by the addition of a carbon atom [7]. In this study, we performed a more detailed structure–activity study of the inhibition of choline uptake by choline analogues. While we did not examine the transport of the analogues, only their inhibition of choline transport, this data should still provide some idea of the active site of the carrier. Our results are in general agreement with these earlier hypotheses.

METHODS

Choline uptake studies were performed as described by Simon *et al.* [11] with certain minor modifications. Uptake reactions were begun by adding [^{3}H]choline and the inhibitor to synaptosomal preparations in Krebs–Ringer buffer that had been preincubated for 5 min at 37°. The final concentration of [^{3}H]choline was 0.6 μM. After a 4-min incubation period, the reactions were terminated by the addition of 2 ml of ice-cold Krebs–Ringer medium. Uptake activity was calculated as described previously [11].

The data were analyzed by log-probit analysis and presented as IC_{50} (concentration required to produce 50 per cent inhibition) values [2]. Experiments were repeated three times, and IC_{50} values varied by less than 15 per cent.

Choline analogues were either purchased from standard supply sources or synthesized by standard procedures and were characterized by spectroscopic methods. Compounds 2–5 were prepared by the addition of the appropriate alkyl iodide with *N,N*-dimethylethanolamine. Compounds 9–12 and 15–17 were prepared by the substitution of the appropriate halogen analog by trimethylamine. Compounds 14 and 24 were prepared by the quaternarization of the respective amines with methyl iodide. Other details are available on request.

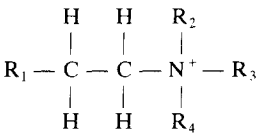
RESULTS

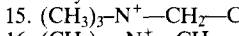
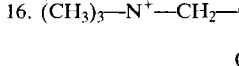
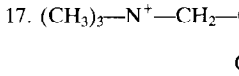
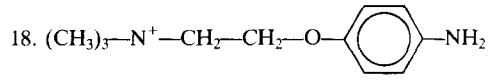
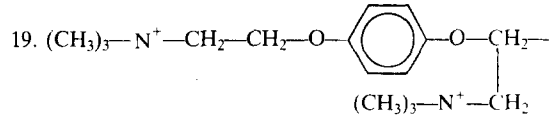
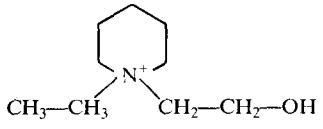
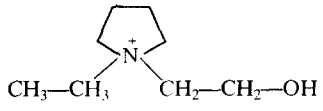
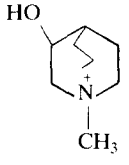
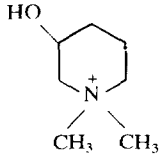
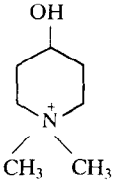
The inhibition of [^{3}H]choline uptake by twenty-four compounds was examined. Their IC_{50} values were determined as described in Methods (see Table 1).

A number of compounds were tested which had changes in the substituents on the quaternary nitrogen (see compounds 1–8, 20 and 21). When using unlabeled choline to obtain an IC_{50} value for itself (actually its K_T) we found a value of 0.63 μM. When changes were made on the quaternary nitrogen, all of the compounds tested showed a loss of potency, although some were still quite potent. Substitution

‡ Author to whom all correspondence should be addressed.

Table 1. Structures of some compounds used (referred to on left by number)



Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)
1. Choline	OH	Ch_3	Ch_3	Ch_3	0.63 (K_T)
2. *N*-Ethylcholine	OH	CH_3	CH_3	C_2H_5	1.4
3. *N*-Propylcholine	OH	CH_3	CH_3	$(CH_2)_2CH_3$	1.9
4. *N*-Isopropylcholine	OH	CH_3	CH_3	$CH(CH_3)_2$	5
5. *N*-Butylcholine	OH	CH_3	CH_3	$(CH_2)_3CH_3$	16
6. *N*-Benzyl-*N*,*N*-diethylaminoethanol	OH	C_2H_5	C_2H_5	C_7H_7	30
7. *N*-Benzylcholine	OH	CH_3	CH_3	C_7H_7	41
8. *N*-Butyl-*N*,*N*-diethylaminoethanol	OH	C_2H_5	C_2H_5	C_4H_9	44
9. Chlorocholine	Cl	CH_3	CH_3	CH_3	18
10. Fluorocholine	F	CH_3	CH_3	CH_3	12
11. *O*-Methylcholine	OCH_3	CH_3	CH_3	CH_3	153
12. *O*-Ethylcholine	OC_2H_5	CH_3	CH_3	CH_3	160
13. Homocholine	CH_2OH	CH_3	CH_3	CH_3	2.2
14. Butylcholine	CH_2CH_2OH	CH_3	CH_3	CH_3	310
15. 					100
16. 					20
17. 					450
18. 					10
19. 					0.7
20. 					6.8
21. 					1.6
22. 					1.4
23. 					22
24. 					48

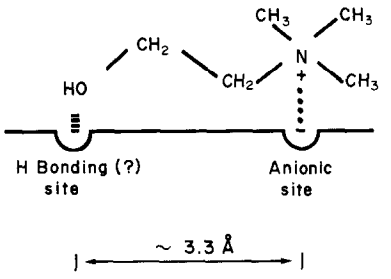


Fig. 1. Schematic of tentative choline site on the high affinity carrier. See text for discussion.

with an ethyl or a normal-propyl resulted in a 3-fold change in the IC_{50} value. Compound 21 was also fairly potent even though the quaternary nitrogen had both a ring structure and an ethyl group. All of the other compounds tested showed a greatly reduced potency.

A number of compounds with substitutions at the hydroxyl part of the molecule were tested (Compounds 9–12 and 15–18). None of these compounds were potent inhibitors. Thus, halogen substitution, the formation of ethers, lengthening the carbon chain, or the production of other alterations resulted in a weak inhibition of uptake. Compound 19, a bis-quaternary compound, stood out as a surprisingly good inhibitor.

Other compounds with changes in the hydroxyl to quaternary nitrogen distances, as well as with other changes, were examined (Compounds 13, 14, 22 and 24). The sharp loss in potency between Compounds 13 and 14 suggests that there are limits to how far one can separate the hydroxyl group from the quaternary nitrogen. The striking difference in potency between Compounds 22 and 23 supports this idea. Based on crystallographic studies of the choline molecule [12, 13], one might conclude that the nitrogen–oxygen (N–O) distance in the preferred conformation is about 3.26 Å (Fig. 1). We used Dreiding molecular models to obtain a semi-quantitative measure of the N–O distance in the other compounds. Compound 22, the rigid 3-quinuclidinol methiodide, has an N–O distance of about 3.4 Å, while Compound 23, the piperidinol derivative, has a significantly larger N–O distance of 3.7–3.8 Å (assuming the more stable 'chair' form of No. 23). Compound 24 has an even larger N–O distance and less inhibitory potency (Table 2). Compounds 22 and 23 are racemic mixtures and one could resolve them into the *d* and *l* forms. It is quite possible that one of the forms would be a better inhibitor than the other.

Table 2. Comparison of nitrogen–oxygen distance and uptake inhibitory potency for some compounds

Compound	Nitrogen–Oxygen distance* (Å)	Choline uptake IC_{50}† (μM)
Choline	3.26	$K_T = 0.63$
22	3.4	1.4
23	3.7–3.8	22
24	4.1	48

* Data are from crystallographic studies (choline) [12, 13] and from estimations with Dreiding models. See text.

† See Table 1 and Fig. 1.

DISCUSSION

The results found here are in general agreement with earlier observations in experiments with both brain synaptosomes and T-sacs from *Torpedo* [2–4]. Not only is there a requirement for a quaternary nitrogen [2], but there appears to be some size limitation on the nitrogen substituent. The larger the substituent, the less effective the compound is as an inhibitor. There did not seem to be any particularly strong or sharp cutoff point in that series, although potency dropped off quickly after *n*-propylcholine. Also in agreement with earlier studies [2], our results indicate that a hydroxyl group is quite necessary for inhibition of uptake (the bis-quaternary compounds are an exception to this; see below). No tested substitution resulted in a good inhibitor. With regard to the length of the carbon chain between the quaternary nitrogen and the hydroxyl group, there appears to be some limitation. Going from three carbons (homocholine) to four carbons resulted in a 100-fold loss of inhibitory potency. Thus, while there appears to be some flexibility in the length of the carbon chain, it apparently cannot be any greater than three carbons.

Even though a compound may be a good uptake inhibitor in this study, we have no way of knowing with the present data whether or not it can be a substrate for the carrier. In the past, the approach taken to test this was to synthesize radioactive choline analogues and test for uptake, acetylation and release [5–10]. Several compounds have been found to be substrates for this system and to be false neurotransmitters [5–10].

Compound 19 appeared to be an unusually good uptake inhibitor. The reasons for this are unclear; however, it has been reported that compounds with two quaternary nitrogens separated by carbon chains are very potent inhibitors of uptake [14]. In fact, compounds in this series can be as potent as homicholinium-3 and some 50–100 times more potent than choline itself [14, 15]. There is no evidence that bis-quaternary compounds are transported.

By comparing estimated N–O distances for some compounds from crystallographic data [12, 13] and Dreiding models, one arrives at a tentative N–O distance of about 3.3 Å for a fit to the carrier. Compounds with a larger N–O distance become poor inhibitors of uptake, presumably because of a poor fit with the carrier. Homocholine, a compound that is a substrate for the carrier [6, 7], can fold to the postulated N–O distance of about 3.3 Å. Further studies would be required to substantiate these ideas.

Lastly, these and other results permit some tentative description of the active site of the carrier. The requirement for a quaternary nitrogen implies the existence of an anionic site on the carrier for electrostatic attraction. The length of the side chain carbons on the quaternary nitrogen is important and it cannot get too large. The requirement for a hydroxyl group suggests a possible requirement for

hydrogen bonding. The distance between the presumed anionic site and the hydroxyl site is possibly of the order of 3.3 Å. Given this data, the carrier seems optimally designed for choline itself.

While choline transport has been studied in other tissues such as kidney slices [16] and erythrocytes [17], one cannot assume that our findings with brain will apply to these other tissues. Rather, there is evidence that the carriers are different. For example, choline uptake by erythrocytes appears to have a 10-fold higher K_T for choline [17] and a different rank order of inhibitory potencies for analogues [18] compared to the high affinity uptake for brain.

Acknowledgements—The authors acknowledge the clerical assistance of Ms. Darlene Weimer and the support of the McKnight Foundation and USPHS Grants MH25951 and MH00053.

REFERENCES

1. M. J. Kuhar and L. C. Murrin, *J. Neurochem.* **30**, 15 (1978).
2. J. R. Simmon, T. Mittag and M. J. Kuhar, *Biochem. Pharmac.* **24**, 1139 (1975).
3. M. J. Dowall, in *Advances in Behavioral Biology* (Ed. D. J. Jenden), Vol. 24, pp. 359–378. Plenum Press, New York (1978).
4. M. J. Dowdall, F. J. Barranter, W. Stendee and T. M. Jovin, *J. Neurochem.* **27**, 1253 (1976).
5. L. A. Barker, T. W. Mittag and B. Krespan, in *Cholinergic Mechanisms and Psychopharmacology* (Ed. D. Jenden) pp. 465–480. Plenum Press, New York (1978).
6. L. A. Barker and T. W. Mittag, *J. Pharmac. exp. Ther.* **192**, 86 (1975).
7. L. A. Barker and T. W. Mittag, *Biochem. Pharmac.* **25**, 1931 (1976).
8. B. Collier, S. Lovat, D. Alson, L. A. Barker and T. W. Mittag, *J. Neurochem.* **28**, 331 (1977).
9. B. Collier and D. Ilson, *J. Physiol., Lond.* **246**, 489 (1977).
10. C. V. Chiou, *Life Sci.* **14**, 1721 (1974).
11. J. R. Simon, S. Atweh and M. J. Kuhar, *J. Neurochem.* **26**, 909 (1976).
12. M. E. Senko and M. Templeton, *Acta crystallogr.* **13**, 281 (1960).
13. F. G. Canepa, *Nature. Lond.* **207**, 1152 (1965).
14. J. T. Holden, J. Rossier, J. C. Beaujovan, P. Guarjenet and J. Glowinski, *Molec. Pharmac.* **11**, 19 (1975).
15. P. Guyenet, P. Lefresne, J. Rossier, M. C. Beaujovan and J. Glowinski, *Molec. Pharmac.* **9**, 630 (1973).
16. C.P. Sung and R. M. Johnstone, *Can. J. Biochem.* **43**, 1111 (1965).
17. K. Martin, *J. gen Physiol.* **51**, 497 (1968).
18. K. Martin, *Br. J. Pharmac.* **36**, 458 (1969).