Biochemical Pharmacology, Vol. 24, pp. 1763–1766. Pergamon Press, 1975. Printed in Great Britain.

[^{3}H]CHOLINE ENTRY AND [^{3}H]ACETYLCHOLINE FORMATION IN LEECH SEGMENTAL GANGLIA

Š. ADAMIČ

Institute of Pathophysiology, Medical Faculty, University of Ljubljana, 61105 Ljubljana, Yugoslavia

(*Received* 19 *December* 1974; *accepted* 20 *March* 1975)

Abstract—Choline entry into the cells of segmental ganglia of the leech, *Hirudo medicinalis* was studied. Kinetic data obtained for two different choline concentration ranges suggest that there are two different Michaelis constants for the carrier-mediated choline entry, one about 860 μM and the other about 45 μM. The percentage of labelled choline transformed into acetylcholine was considerably higher if the ganglia were incubated in 6 μM [^{3}H]choline than in 250 μM [^{3}H]choline. Hemicholinium-3 at 10 and 100 μM inhibited choline entry rate and diminished the percentage of labelled choline transformed into acetylcholine. Removal of sodium from the incubating medium affected the choline entry to roughly the same degree at low and at high choline concentrations. The percentage of labelled choline transformed into acetylcholine did not diminish in the sodium-free medium. The results might be interpreted in terms of two choline entry systems in leech segmental ganglia. The choline entry system with the high affinity seems to be linked to acetylcholine synthesis.

The entry of choline into cells is important for phospholipid as well as acetylcholine synthesis. In connection with the latter process the entry of choline into cells of the central nervous system has been investigated by many authors. Recently Yamamura and Snyder [1, 2] showed that in rat brain synaptosomes two systems of choline entry exist, one with a low and the other with a high affinity for choline. It has also been shown that the high-affinity choline entry system is associated with a marked degree of acetylcholine formation [1–3].

In our previous work the mechanism of the choline entry into the cells of segmental ganglia of the leech was studied [4]. It was found that choline entry obeyed the Michaelis–Menten kinetics and it was suggested that these ganglia might be used for the study of choline movement across the cell membranes of the central nervous system. The aim of the present work was to find out whether also in the segmental ganglia of the leech a separate choline entry mechanism linked to acetylcholine synthesis could be detected.

METHODS

The experiments were carried out on segmental ganglia isolated from the leech *Hirudo medicinalis*. Leeches weighing 1·5–2 g were anesthetized in 10% ethanol. The central nervous system was exposed under a dissecting microscope, the bilateral nerve roots were dissected and samples of the ganglionic chain consisting of four segmental ganglia together with their connectives were prepared. The samples were incubated in 20 μl of incubating solution to which radioactive choline with a specific radioactivity of about 5 μCi/ml and, whenever necessary hemicholinium-3 (HC-3) were added. At higher choline concentrations non-radioactive choline was also added in order to obtain the desired initial concentration. As a rule leech Ringer solution modified by Nicholls and Kuffler [5], containing 115 mM NaCl, 4 mM KCl, 1·8 mM $CaCl_2$, 10 mM Tris, 10 mM maleic acid, 10 mM NaOH, 11 mM glucose, pH 7·4, was used for incubation. In some experiments a sodium-free incubating solution of the following composition was used: 250 mM sucrose, 4 mM KCl, 1·8 mM $CaCl_2$, 10 mM Tris, 5 mM maleic acid, 11 mM glucose; pH 7·4. After incubation the ganglionic chain was quickly rinsed with leech Ringer solution, blotted with filter paper and the connectives removed. The ganglia were solubilized overnight in 10 μl of 1 N NaOH and prepared for radioactivity measurements.

The percentage of labelled choline and acetylcholine obtained in ganglia after incubation in labelled choline, was determined by paper chromatography. The technique used was generally the same as that described previously [4] except that acetylcholine hydrolysis was prevented by tetraethylpyrophosphate (TEPP) instead by DFP.

The intracellular concentration of the radioactive material in the ganglia after incubation in labelled choline was calculated using the data on the total radioactivity in the ganglia obtained in each particular experiment, as well as the data on the ganglion weight, extracellular space and water content obtained in our previous work [4].

[*N-methyl*-^{3}H]choline chloride, sp. act. 17 Ci/mmole, was purchased from the Radiochemical Centre, Amersham and stored at 4°. To make sure that during storage no chemical decomposition occurred, the stock solution of radioactive choline was periodically checked by paper chromatography using the same solvent system as referred to above.

Radioactivity was measured by liquid scintillation spectrometry (Unilux II, Nuclear Chicago) using a modified Bray's liquid scintillation mixture containing 5 g diphenyloxasole (PPO), 0·5 g *p*-bis-[2-(5-phenyloxasolyl)] benzene (POPOP) and 80 g naphthalene per litre of solvent consisting of equal volumes of toluene, *p*-dioxane and ethyleneglycol monomethyl ether.

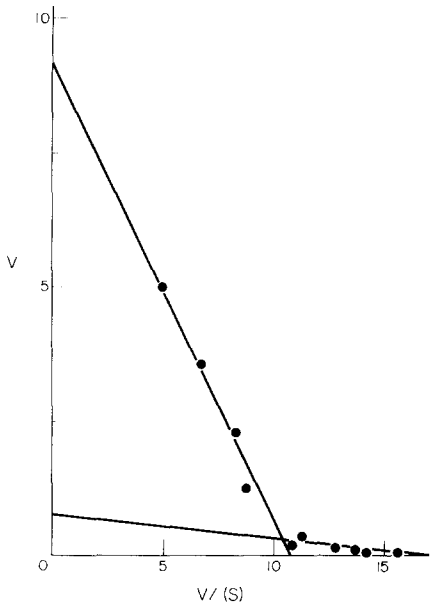

Fig. 1. The dependence of the rate of choline entry into the segmental ganglia of the leech on the external choline concentration. Plot of v against $v/[s]$. v, in m-moles/l. of intracellular water per 30 min; $[s]$, in mM.

RESULTS AND DISCUSSION

In our previous work [4] choline entry into leech segmental ganglia at choline concentrations between 0·06 mM and 1 mM was found to obey the Michaelis Menten kinetics with the Michaelis constant of about 1 mM. In the present work we first tried to check whether the same Michaelis constant would be obtained if the choline entry rate was measured within a lower choline concentration range. For this purpose the choline entry into segmental ganglia incubated in labelled choline was studied within two different concentration ranges. The results plotted as v against $v/[s]$ are shown in Fig. 1. The curve obtained can be resolved in two components. The steeper component represents the relationship between v and $v/[s]$ within the choline concentration range 0·15 mM 1 mM. The maximum entry rate and the Michaelis constant obtained from this component were found to be about 9 m-moles/l. of intracellular water per 30 min and 0·86 mM, respectively. The value of each of these two parameters is roughly the same as that obtained in our previous work for the same concentration range [4]. The flatter component of the curve represents the relationship between v and $v/[s]$ with the choline concentration range 3–30 μM. The maximum entry rate and the Michaelis constant obtained from this component were found to be about 750 μmoles/l. of intracellular water per 30 min and 45 μM, respectively. Thus, the value of the two parameters is 10 20 times higher when measured within the lower choline concentration range.

Qualitatively similar results were obtained in experiments with the rat brain synaptosomes [2, 3], and interpreted in the sense that there were two choline entry systems, one with a low affinity and the other with a high affinity for choline. The two values of the Michaelis constant found for our preparation are, however, roughly by one order of magnitude higher than those found for synaptosomes [2]. This difference is probably due to the considerable difference between the two preparations. Different experimental conditions might also influence the results as different authors working with rat synaptosomes obtained different values for the Michaelis constant [2, 3, 6].

In another series of experiments the synthesis of acetylcholine from the labelled choline in ganglia incubated at different choline concentrations, was studied using paper chromatography. When ganglia were incubated in labelled choline at the concentration of 250 μM, only about 10% of the radioactive material in the ganglia was found to be acetylcholine. However, when ganglia were incubated at the choline concentration of 6 μM, the labelled acetylcholine represented about 30% of the total radioactive material (Fig. 2).

The percentage of the labelled acetylcholine in ganglia incubated in tritiated choline at the concentration at which the high affinity choline entry component predominated, was higher than at the choline concentration within the range of the low affinity component. Similar results had been obtained by Yamamura and Snyder [1, 2] as well as Haga and Noda [3] and explained in the sense that the high affinity choline entry is connected with acetylcholine synthesis.

In another series of experiments the effect of HC-3 on choline entry and acetylcholine synthesis, was studied. The results showed that at 6 μM external choline concentration HC-3 lowered the choline entry rate. If 10 μM HC-3 was used, about 25% of the choline entry was inhibited whereas if HC-3 concentration was increased to 100 μM, an inhibition of about 70% was obtained. In separate experiments the influence of these two concentrations of HC-3 on the

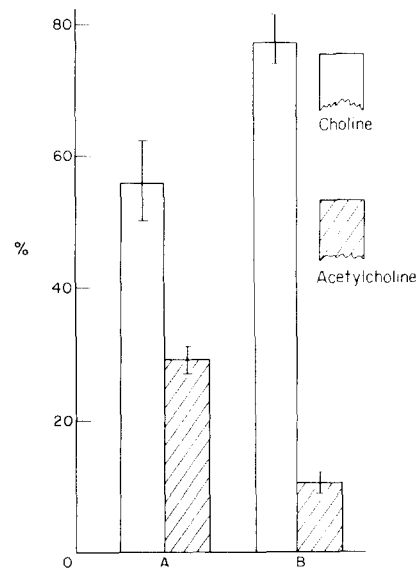

Fig. 2. The synthesis of labelled acetylcholine in leech ganglia incubated for 30 min at room temperature in leech Ringer solution with labelled choline. Percentage of the total amount of the radioactive material in the ganglia. (a), 6 μM external choline concentration (n = 6); (b), 250 μM external choline concentration (n = 6).

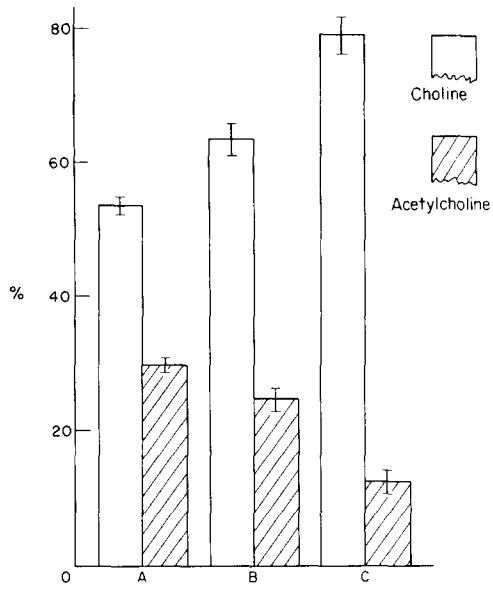

Fig. 3. The influence of hemicholinium-3 on the synthesis of labelled acetylcholine in leech ganglia incubated for 30 min at room temperature in leech Ringer solution containing 6 μM tritiated choline. Percentage of the total amount of the radioactive material in the ganglia. (a), control experiments (n = 9); (b), 10 μM hemicholinium-3 added (n = 9); (c), 100 μM hemicholinium-3 added (n = 9).

transformation of the entered choline into acetylcholine, was studied. The percentage of the radioactive material in the form of acetylcholine in the ganglia previously incubated in 6 μM labelled choline was determined by paper chromatography. The results are presented in Fig. 3. The percentage of labelled acetylcholine in the ganglia decreased and the percentage of labelled choline increased if HC-3 was added to the incubating medium. This inhibitory effect increased from 17% to about 60% if the HC-3 concentration was increased from 10 to 100 μM. Similarly, an inhibition of both choline entry and acetylcholine synthesis by HC-3 was found to occur in rat brain synaptosomes [3, 6]. A correlation between the two inhibitory effects was also observed [7].

In a further series of experiments the dependence of choline entry on the sodium concentration in the incubating solution was studied. The results of these experiments are presented in Table 1. The rate of choline entry into the ganglia incubated in the sodium-free medium was reduced to almost half its control value. However, the effect of sodium removal on the choline entry rate was the same irrespective of whether 250 or 3 μM choline was used. In order to see whether the transformation of labelled choline into acetylcholine is affected by sodium removal, the percentage of labelled choline and acetylcholine in the radioactive material obtained from the ganglia after incubation in labelled choline, was determined by paper chromatography. The results presented in Fig. 4 show that there was no decrease in the percentage of the labelled acetylcholine in the radioactive material in the ganglia incubated in the sodium-free solution. It seems, therefore, that in the case of leech ganglia the carrier-mediated choline entry is sodium-

Table 1. The effect of a sodium-free solution on the choline entry rate at different external choline concentrations

Choline (μM)	a_i/a_e		
	Control	Na-free	% inhibition
3	13·87 ± 1·2	7·90 ± 0·4	57
250	7·30 ± 0·7	3·76 ± 0·4	52

Parts of the ganglionic chain were incubated for 30 min at room temperature in leech Ringer solution with tritiated choline. a_i/a_e is the ratio between the sp. act. of intracellular water and of the incubating solution. Mean values of ten experiments (±S.E.).

dependent but that the effect of sodium on both the high and the low affinity choline entry system is equal. A dependence of choline entry on sodium in the incubating medium has been shown with neuroblastoma cells [8] and with brain synaptosomes [2, 3]. With the latter preparation, however, the high-affinity choline entry system, linked to acetylcholine synthesis is more potently affected by sodium removal than is the low affinity system. The sodium dependence of the choline entry systems in leech segmental ganglia seems to differ from that described for rat brain synaptosomes.

Our results might be interpreted in the sense that there are two distinct systems of the choline entry into leech ganglia cells, one with a low and the other with a high affinity for choline. The fact that at low choline concentrations, when, the high-affinity entry system predominates, a larger percentage of labelled choline is transformed into acetylcholine than at high

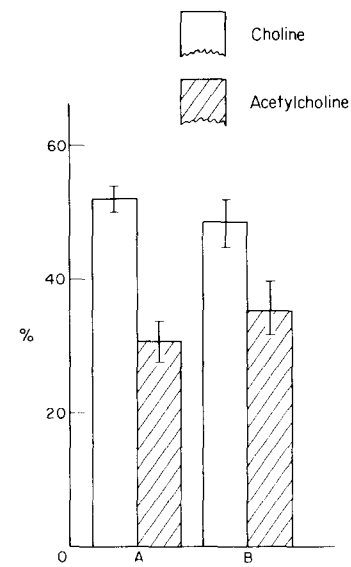

Fig. 4. The influence of a sodium-free solution on the synthesis of labelled acetylcholine. Leech ganglia were incubated for 30 min at room temperature in either normal or sodium-free leech Ringer solution containing 6 μM tritiated choline. After incubation the radioactive material in ganglia was identified by paper chromatography. Percentage of the total amount of the radioactive material in the ganglia. (a), normal leech Ringer solution (n = 8); (b), sodium-free solution (n = 8).

choline concentrations, might be explained in terms of a specific connection between the high-affinity system and intracellular acetylcholine synthesis. It should be stressed that though the percentage of labelled choline transformed into acetylcholine was relatively high at lower choline concentrations, on the absolute scale, the rate of labelled acetylcholine formation was lower because of the lower choline concentration. The effects of HC-3, which at 10 μM and 100 μM concentrations reduced the choline entry rate and diminished the percentage of choline transformed into acetylcholine, might be explained in terms of a selective action of HC-3 on the high-affinity choline entry system. Our results are in agreement with those obtained for rat brain synaptosomes [6]. The possibility that the activity of choline acetyltransferase might be affected by HC-3 could not be avoided in our experiments. The fact that the sodium-free solution affected both systems of choline entry to roughly the same degree suggests that in leech ganglia both systems are sodium-dependent. On the other hand, the transformation of choline into acetylcholine does not seem to be sodium-dependent since the percentage of labelled acetylcholine formed from choline entering the ganglia, did not diminish in the sodium-free solution. These findings are rather different from those obtained for synaptosomes [2, 3].

It might be expected that the choline entry system linked to acetylcholine synthesis is located mainly in the cholinergic nervous system. When rat brain synaptosomes were incubated in choline at a concentration of 10 μM or lower, cholinergic nerve endings were found to be responsible for the bulk of the accumulated choline [9]. So far, the cholinoreceptive system in the leech ganglia has not been extensively studied whereas more attention has been paid to some non-cholinergic mediators. However, the presence of acetylcholine in the leech nervous system was shown many years ago [10], the spontaneous activity of the cells of Retzius was found to increase following the application of acetylcholine [11], and the activity of choline acetyltransferase was found to be considerably higher in leech ganglia than in the connectives of the ganglionic chain [12]. These observations point to the existence of a cholinergic nervous system in leech ganglia, though detailed data on its localization and role are still lacking. The existence of a choline uptake system linked with acetylcholine synthesis supports such a view.

Acknowledgements—This work was supported by the Boris Kidrič Foundation, Ljubljana and by the NIH grant No. 02-008-1 Z-ZF-6. The technical assistance of Mrs. Marjeta Dobovišek is gratefully acknowledged.

REFERENCES

1. H. I. Yamamura and S. H. Snyder, *Science, N.Y.* **178**, 626 (1972).
2. H. I. Yamamura and S. H. Snyder, *J. Neurochem.* **21**, 1355 (1973).
3. T. Haga and N. Noda, *Biochim. biophys. Acta* **291**, 564 (1973).
4. Š. Adamič, *Biochem. Pharmac.* **23**, 2595 (1974).
5. J. G. Nicholls and S. W. Kuffler, *J. Neurophysiol.* **27**, 645 (1964).
6. J. G. Nicholls and S. W. Kuffler, *Molec. Pharmac.* **9**, 630 (1973).
7. P. Guyenet, P. Lefresne, J. Rossier, J.-C. Beaujouan and J. Glowinski, *Brain Res.* **62**, 523 (1973).
8. R. Massarelli, J. Ciesielski-Treska, A. Ebel and P. Mandel, *Pharmac. Res. Commun.* **5**, 397 (1973).
9. M. J. Kuhar, V. H. Sethy, R. H. Roth and G. K. Aghajanian, *J. Neurochem.* **20**, 381 (1973).
10. A. Carayon-Gentil and J. Gautrelet, *C. r. Séanc. Soc. Biol.* **127**, 887 (1938).
11. G. A. Kerkut and R. J. Walker, *Br. J. Pharmac.* **30**, 644 (1967).
12. B. A. Perkins and G. A. Cottrell, *Comp. gen. Pharmac.* **3**, 19 (1972).