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EFFECTS OF QUATERNARY AMMONIUM COMPOUNDS ON CHOLINE ENTRY INTO THE RAT DIAPHRAGM MUSCLE FIBRE*

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Abstract—The mechanism was studied of the inhibition of entry of choline (*N*-methyl-^{14}C) into the rat diaphragm muscle fibre by some quaternary ammonium compounds, Hemicholinium (K_i = 0·16 mM), tetraethylammonium (K_i = 56 mM), tetramethylammonium (K_i = 10 mM), *d*-tubocurarine (K_i = 2·2 mM) and decamethonium (K_i = 3·7 mM) competitively inhibited choline entry, whereas hexamethonium in concentrations up to 50 mM showed no inhibitory effect. The results suggest that the choline carrier has a higher affinity for the quaternary ammonium compounds which are known as neuromuscular blocking agents than for those which are known as ganglionic blocking agents.

IN RECENT years it has been found that choline is concentrated by many types of tissue, e.g., by the mouse cerebral cortex,[1] the squid axon,[2] the synaptosomes prepared from the guinea-pig cerebral cortex,[3,4] the kidney,[5] the human erythrocytes,[6] the cardiac muscle of the cat,[7] and the rat diaphragm muscle fibre.[8,9] Some of these experiments designed to explain the mechanism of the choline entry, have shown that the choline influx is mediated by a carrier, the nature of which is so far unknown. It has also been shown that the carrier can be inhibited by some substances mainly those which are known to play a role in the cholinergic nervous mechanism. Though such inhibitory effects have been observed in several types of cells, there is a lack of data concerning the type of inhibition and the affinity of the choline carrier for the substances that have been studied. It seems, however, that such data might be rather important for the study and comparison of the structures of the choline carrier found in various tissues.

Our previous studies have shown that the choline entry into the rat diaphragm muscle fibre is mediated by a carrier[8] and that this process is inhibited by acetylcholine.[10] The aim of the present work is to investigate the inhibition of choline entry into the rat diaphgram muscle fibre by some quaternary ammonium compounds known to interact also with the choline carrier of some other tissues.

METHODS

The experiments were carried out on isolated hemidiaphragms of albino rats of both sexes, weighing from 100–150 g. The diaphragm was excised under ether anaesthesia and placed in the incubating solution (Krebs bicarbonate buffer with 200 mg of glucose/100 ml, pH 7·4) at room temperature. The dorsal part of the diaphragm,

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the superficial connective tissue and all but a small portion of the rib and of the intercostal muscle tissue were removed under a dissecting microscope. The hemidiaphragm with the rest of the ribs and of the central tendon, was incubated at 38° by gently shaking in 6 ml of incubating solution containing radioactive choline (0·05 μc/ml), nonradioactive choline in amounts necessary to obtain the desired initial concentration, and the inhibitory substance tested. In parallel control experiments the inhibitory substance was omitted. After incubation, the hemidiaphragm was rinsed with saline and blotted. Two strips (approx. 30 mg) of undamaged diaphragm muscle without the tendon and ribs were excised, weighed and dissolved in 0·5 ml of hot 1 N NaOH. Subsequently, samples of the dissolved muscle and of the incubating medium were prepared for radioactivity measurement. The water content was determined by drying the muscle to the constant weight. The inulin extracellular space was determined using the method of Roe *et al.*[11]

Choline (*N*-methyl-^{14}C) with a specific radioactivity of 50 mc/mmole, purchased from the Radiochemical Centre, Amersham, was eluted from the paper with water and stored at −30°. To make sure that the radioactive material was still in the form of choline, the stock solution of the radioactive choline was periodically checked by paper chromatography using the following solvent system; *n*-butanol–ethanol–acetic acid–water (8:2:1:3, by vol.).

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

The choline entry rate was calculated and expressed in millimoles of choline entering 1 l. of the intracellular water per hr.

RESULTS

In order to minimize the effect of the non-saturable component of the choline entry, only choline in concentrations of 0·2 mM or lower, was used in our experiments.

Of all substances tested in our experiments hemicholinium-3 (HC-3) was found to be the most potent inhibitor of the choline entry. The inhibition was of the competitive type (Fig. 1A) and the inhibitor constant was found to be about 0·16 mM. In order to find out whether HC-3 at lower concentrations stimulated choline entry, a fact observed with synaptosomes,[12] the effect of HC-3 at concentrations ranging from 0·1 mM to 0·01 μM was also studied. However, no stimulatory effect on choline entry was found in this concentration range of HC-3.

Of the monoquaternary ammonium compounds, tetraethylammonium (TEA) and tetramethylammonium (TMA) were used. Each of the two substances competitively inhibited choline entry (Fig. 1B), TMA being a stronger inhibitor than TEA. The inhibitor constant of TMA and that of TEA have been found to be 10 and 56 mM, respectively.

In further experiments, the effect of some bisquaternary ammonium compounds on choline entry was studied. Decamethonium (C-10) competitively inhibited the choline entry into the rat diaphragm muscle fibre (Fig. 1C). The inhibitor constant was found to be 3·7 mM. On the other hand, hexamethonium (C-6) at concentrations

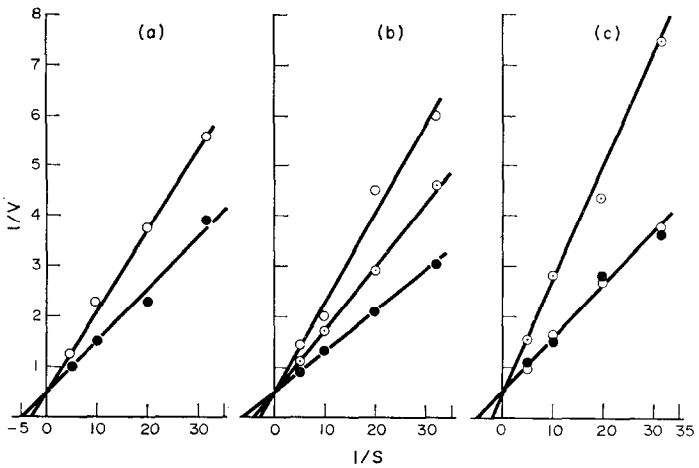

FIG. 1. Lineweaver–Burk plot of inhibition of choline entry by quaternary ammonium compounds. Rat diaphragms were incubated for 40 min at 38° in Krebs bicarbonate buffer with different choline concentrations without inhibitor ●——●, or with the following substances added: (A) 0·1 mM hemicholinium-3 ○——○; (B), 10 mM tetramethylammonium ○——○; or 20 mM tetraethylammonium ⊙——⊙; (C), 50 mM hexamethonium ○——○; or 5 mM decamethonium ⊙——⊙. s = mM, v = mmoles/l of intracellular water/hr.

up to 50 mM showed no significant effect on choline entry. Since already at these concentrations either the ionic strength or the ionic composition of the incubating solution must be changed, as a consequence of which nonphysiological experimental conditions are obtained, higher concentrations of C-6 were not used and the inhibitor constant for C-6 was not determined. It is obvious, however, that the affinity of the choline carrier for C-6 is much lower than it is for C-10. D-tubocurarine (d-TC), another bisquaternary ammonium compound, showed an inhibitory effect very similar to that of C-10. The inhibition of the choline entry was competitive and the inhibitor constant 2·2 mM.

DISCUSSION

The inhibitor constants of substances tested in our experiments are presented in Table 1. The strongest inhibition was obtained with HC-3, a fact observed also in experiments with some other tissues.[13,14] Nevertheless, the inhibitor constant of HC-3 for the choline carrier in the rat diaphragm muscle fibre is about 50 times higher than that for the choline carrier in human erythrocytes[13] and roughly 5 times higher than that for the carrier in synaptosomes from the guinea-pig brain.[14]

The inhibitor constant of TMA and that of TEA for the choline carrier are by one order of magnitude higher than those for the choline carrier found in erythrocytes.[13] However, both in our experiments and in the experiments with erythrocytes, the affinity of the carrier was higher for TMA than it was for TEA. This fact points to a similarity between these two processes and to a difference between the choline entry mechanism in our tissue preparation and that in synaptosomes since in the latter the choline carrier showed roughly the same affinity for both TEA and TMA.[12] It is interesting to note that the actylcholine carrier in mouse brain slices has even a higher affinity for TEA than it has for TMA,[15] though it should be kept in mind that

TABLE 1. INHIBITOR CONSTANTS OF SOME QUARTERNARY AMMONIUM COMPOUNDS FOR THE CHOLINE ENTRY INTO THE RAT DIAPHRAGM MUSCLE FIBRE

Compound	Inhibitor constant (mM)
Hemicholinium-3	0·16
Tetramethylammonium	10
Tetraethylammonium	56
Decamethonium	3·7
Hexamethonium	>50
D-tubocurarine	2·2

in the brain the mechanisms of the acetylcholine entry probably differs from that of the choline entry.[16] There is a similar difference in the affinity of some other choline- or acetylcholine-binding sites for TMA and TEA. It has been found that, among others, the affinity of the cholinergic receptor in frog muscle increases when in TMA the methyl groups are replaced by ethyl groups,[17] and that also acetylcholinesterase has a greater affinity for TEA than it has for TMA.[18]

The inhibition constants of d-TC and C-10 for the choline carrier in rat diaphragm are rather similar, whereas the constant of C-6 seems to be more than ten times higher. Though these constants for the choline carrier in the rat diaphragm are also higher than those for the choline carrier in some other tissues, the ratio between the affinity of the choline carrier for C-10 and that of the same carrier for C-6 is rather similar to the ratio found with erythrocytes[13] and synaptosomes.[12] In their experiments with the rat diaphragm Taylor *et al.*[19] showed that C-10 entered the muscle fibre and that this process was inhibited by d-TC. The entry rate was higher in the end plate region and the affinity constant of d-TC for the C-10 entry mechanism was estimated[20] to be 0·331 $(\mu M)^{-1}$, which corresponds to an inhibitor constant by three orders of magnitude lower than that of d-TC for the choline carrier in our preparation. These two facts seem to suggest that two different, though similar, mechanisms of C-10 and choline accumulation in the skeletal muscle fibre are involved. The affinity of d-TC for the choline carrier in the rat diaphragm is by several orders of magnitude lower than is its affinity for either cholinoreceptor[21] or the anionic sites of cholinesterase.[22]

Our results show that the affinity of the choline carrier for the neuromuscular blocking agents is higher than it is for the ganglionic blocking agents. This fact suggests a similarity with the choline transport system in erythrocytes and partly with that in synaptosomes. The observation that the inhibitor constants of these substances for the carrier in the rat diaphragm are higher than they are for the carrier in other tissues might be due to a generally lower affinity of the choline carrier in the rat diaphragm, for it was found that K_m for choline in the rat diaphragm was roughly by one order of magnitude higher[8] than that for choline in human erythrocytes,[6] and several times higher than that for choline in synaptosomes.[3,4] The fact that the choline carrier binds quaternary ammonium compounds points to a similarity between the carrier on the one hand and the cholinoreceptors and cholinesterases on the other. Much lower affinities of these substances for the choline carrier, however, suggest that the former is not identical with the latter two.

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