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ACCUMULATION OF ACETYLCHOLINE BY THE RAT DIAPHRAGM*

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Abstract—The mechanism of entry of acetylcholine into the skeletal muscle fibre, was studied. By measuring the uptake of acetylcholine (*N*-methyl-^{14}C) chloride by the rat diaphragm *in vitro* in experiments without tetraethylpyrophosphate, almost all labeled acetylcholine in the medium was hydrolysed and the radioactive material in the muscle was mainly in the form of choline. When tetraethylpyrophosphate was added to the medium, there was still slight hydrolysis, yet the radioactive material in the muscle consisted mainly of acetylcholine. The entry of the labeled substance obeyed the Michaelis–Menten kinetics and had the same maximum velocity as that of choline whereas the apparent Michaelis constant was higher than that for choline. An inhibition of the acetylcholine entry by choline and vice versa, was observed. The results suggest that the entry of acetylcholine is mediated by the same mechanism as the entry of choline, but, that the carrier affinity is lower for acetylcholine than for choline.

THE PASSAGE of acetylcholine (ACh) across the cell membranes has been studied mainly on nervous tissue. When cholinesterase was inhibited by an organophosphate, ACh was found to enter the isolated rat cerebral cortex at a high rate.[1] Further experiments showed that the entry of ACh into the brain cortex slices of the mouse[2] and those of the rat[3] was mediated by a carrier and that the process was blocked in anaerobic conditions.[4, 5]

Our previous work strongly indicates that the choline (Ch) entry into the rat diaphragm muscle fibre might be mediated by a carrier.[6] Creese *et al.*[7, 8] investigating the uptake of some depolarizing drugs by the skeletal muscle, found that iodocholinium as well as decamethonium and carbachol could enter the rat diaphragm muscle fibre. Even though there are a few reports indicating that ACh also might enter the skeletal muscle fibre[9, 10] the mechanism of its entry has not been explored. The aim of the present work was to find out whether ACh is accumulated in the muscle and, if so, to investigate the mechanism of its entry into the rat diaphragm muscle fibre. Several experiments were designed in an attempt to find out whether a high rate of entry into the end-plate region of the muscle fibre could be demonstrated.

METHODS

Albino rats weighing 80–120 g were used. The diaphragm was excised under ether anesthesia 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

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diaphragm, the superficial connective tissue and all but a small portion of rib and intercostal muscle tissue were removed under a dissecting microscope. The diaphragm preparation, with the rest of the ribs and the central tendon, was incubated in 10 ml of the incubating solution containing radioactive ACh (0·05 μc/ml) by gently shaking at 38°. At higher ACh concentrations, the non-radioactive substance was added in order to obtain the desired initial concentration. In the experiments with tetraethylpyrophosphate (TEPP), the diaphragm was preincubated for 5–10 min at room temperature in the incubation solution with TEPP but without ACh. After incubation, the entire diaphragm preparation was rinsed with saline and blotted, the rest of the ribs and the central tendon removed, and the diaphragm muscle weighed and dissolved in 0·5 ml of N NaOH. Subsequently, samples of the dissolved muscle and of the incubation medium were prepared for radioactivity measurement.

In the experiments in which the entry of ACh into the end-plate region was compared with the entry of ACh into the rest of the muscle fibre, the method described by Taylor *et al.*[8] was generally used. At the end of some experiments without TEPP, the end-plates were visualized using Koelle's histochemical method for localizing cholinesterase.[11] In this way a narrow strip containing the end-plates could be separated from the rest of the muscle.

The acetylcholine (*N*-methyl-^{14}C) chloride, supplied by the Radiochemical Centre, Amersham was dissolved in an acetate buffer, pH 4·5, and stored at − 20°.

Radioactivity was determined by liquid scintillation spectrometry (Unilux II, Nuclear Chicago). A modified Bray's liquid scintillation mixture,[12] was used.

The water content was determined by loss in weight after keeping the muscle overnight at 110°. The inulin extracellular space was determined using the method of Roe *et al.*[13]

For the identification of the radioactive material in the muscle tissue extract, descending paper chromatography was used. The tissue was homogenized in cold distilled water adjusted to pH 4–5 with HCl. Subsequently, the extract was heated at 100° for 10 min and the precipitated protein removed by centrifugation. The aqueous extract was applied on chromatographic paper. The chromatogram was allowed to develop for 14–18 hr with the following solvent system: *n*-butanol–ethanol–acetic acid–water (8:2:1:3 by vol.). For radioactivity measurement the chromatogram was cut into small pieces and placed into the liquid scintillation mixture. For the identification of the spots of high radioactivity, a mixture of non-radioactive ACh and Ch was applied on the same chromatogram. After the development, both compounds were visualized by spraying the chromatogram with Dragendorff solution.

RESULTS AND DISCUSSION

A diaphragm incubated with labeled ACh was found to accumulate radioactive material. Figure 1 shows that the rate of the increase in cell radioactivity was much higher in the experiments without TEPP than in those with TEPP. However, in neither set of experiments was equilibrium observed even after 2 hr of incubation. In the experiments without TEPP, the rate of uptake was very similar to that obtained with Ch,[6] and was probably due to the fact that ACh was hydrolysed during the incubation. Since there are different types of esterases in the muscle, it is possible that TEPP does not completely prevent ACh hydrolysis and the low rate of entry of the radioactive material might be due to a lowered rate of ACh hydrolysis.

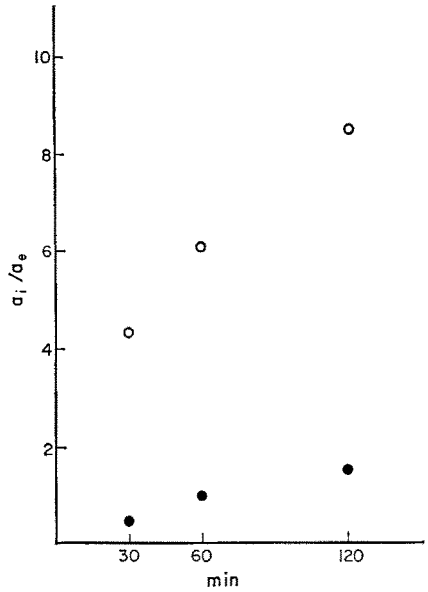

FIG. 1. Time course of acetylcholine uptake by the rat diaphragm. The rat diaphragm was incubated at 38° in Krebs bicarbonate buffer containing labeled acetylcholine at 5 μM concentration without TEPP–○; and with 10 μM TEPP–●. a_i — radioactivity/ml fibre water; a_e = initial radioactivity/ml of medium. The mean values obtained in four to six experiments are presented.

To check this assumption, the chemical nature of the radioactive material found in both the muscle and the medium after incubation under identical experimental conditions was tested by paper chromatography. By this method the R_f values for Ch and ACh were about 0·33 and 0·41, respectively. Though the difference between the two values is not great, the ratio between the amounts of the two substances can be estimated accurately enough. The experiments with TEPP indicate that after 1 hr of incubation in radioactive ACh the medium still contained about 90–94 per cent of ^{14}C in the form of ACh. The rest of ACh was hydrolysed: partly before the experiments, and mostly during incubation. In the same set of experiments about 65 per cent of the radioactive material extracted from the muscle was in the form of ACh, about 30 per cent in the form of Ch, and about 5 per cent in a form whose R_f was about 0·12 (Fig. 2A). It seems, therefore, that in these experiments ACh had entered the cells. However, the radioactive Ch found in the muscle probably resulted from the ACh hydrolysis.

In the experiments without TEPP practically all ACh in the medium was hydrolysed within 1 hr of incubation. In these experiments only about 8 per cent of the radioactive material extracted from the muscle was in the form of ACh, about 68 per cent in the form of Ch and about 24 per cent in the form whose R_f was about 0·12 (Fig. 2B). The R_f of the latter fraction, which was not studied further, was found to be rather close to that of phosphorylcholine,[14] a product of Ch metabolism in some cells.[15] Our experiments show that this fraction was smaller when TEPP was added and seems to depend on the Ch entry. Hence, the high rate of entry of the radioactive material in the experiments without TEPP is probably due to the Ch entry. It seems that the only

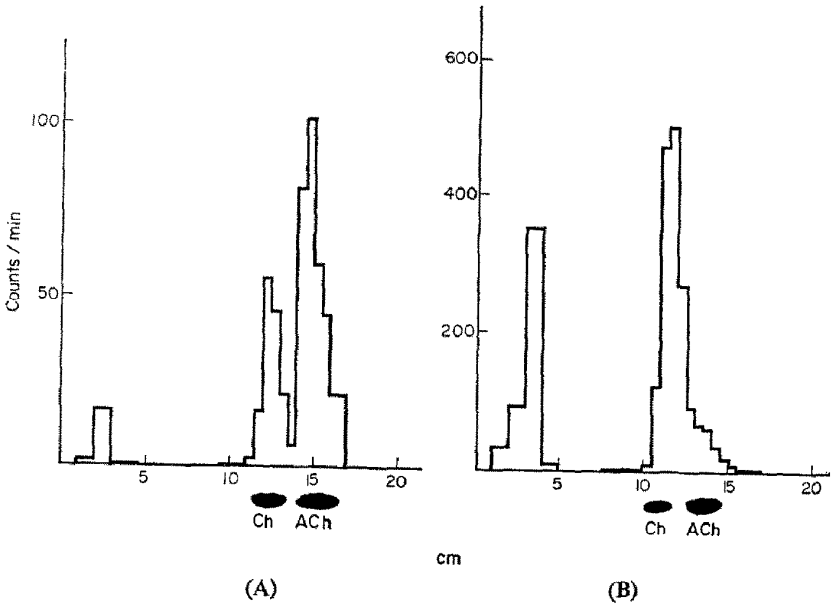

FIG. 2. Paper chromatography of ^{14}C recovered from the rat diaphragm after 1 hr of incubation in labeled acetylcholine with 10 μM TEPP (A) and without TEPP (B). Ch and ACh are spots of unlabeled choline and acetylcholine.

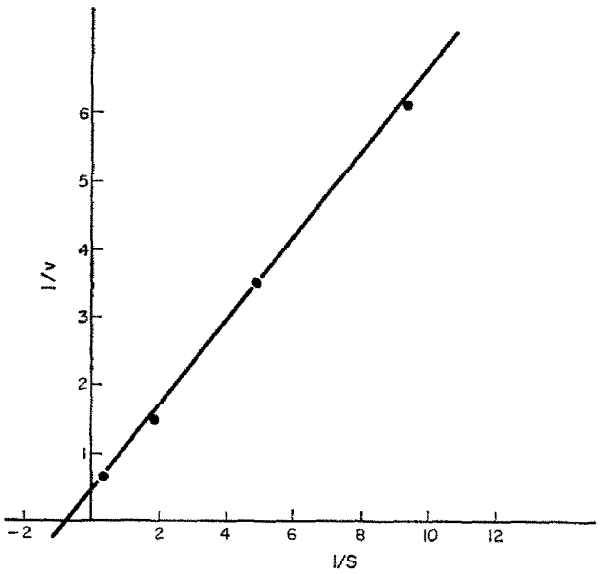

FIG. 3. Lineweaver–Burk plot of acetylcholine uptake in the rat diaphragm. The diaphragm was incubated for 1 hr at 38° in Krebs bicarbonate buffer with acetylcholine at different concentrations and 10 μM TEPP. *s*, in m-moles/1.; *v*, in m-moles/1./hr.

role of TEPP is to prevent ACh hydrolysis, since in some experiments with labeled Ch no significant effect of TEPP on the Ch entry could be demonstrated.

The mechanism of the ACh entry was studied in experiments with TEPP, in which the accumulation of ACh at different concentrations was measured. The results are shown in Fig. 3. The entry of ACh at concentrations from 0·1 to 2·0 mM obeyed the Michaelis–Menten kinetics. The maximum velocity was found to be about 2·8 m-moles/l. of fibre water/hr and the apparent Michaelis constant about 2 mM. The results suggest that the ACh entry might also be mediated by a carrier. Under identical experimental conditions the maximum velocity of the ACh entry and of the Ch entry[6] are equal. The apparent Michaelis constant obtained in our experiments is probably lower than the true Michaelis constant for ACh, for it must be assumed that the former constant is the result of both ACh entry and the simultaneous entry of labeled Ch.

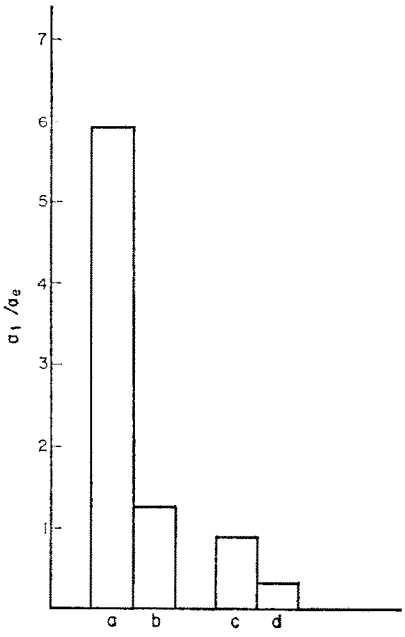

FIG. 4. Mutual inhibition of acetylcholine and choline entry. The rat diaphragm was incubated at 38° for 1 hr in Krebs bicarbonate buffer containing 10 μM TEPP plus labeled and unlabeled substances: a = 5 μM labeled choline, b = 5 μM labeled choline and 10 mM unlabeled acetylcholine, c = 5 μM labeled acetylcholine, d = 5 μM labeled acetylcholine and 10 mM unlabeled choline. a_i = radioactivity/ml of fibre water; a_e = radioactivity/ml of medium.

Our results as well as the chemical structure of the two substances seem to indicate that both the Ch and the ACh entry is mediated by the same carrier. If so, the entry of one substance can be expected to be inhibited by the presence of the other and vice versa. Thus, another set of experiments were designed in order to find out whether such a mutual inhibition between ACh and Ch actually occurs (Fig. 4). The entry of ACh at a 5 μM concentration was considerably smaller when 10 mM non-radioactive

Ch was present. On the other hand, the entry of radioactive Ch at a 5 μM concentration was considerably smaller when 10 mM ACh was added. Since the ACh hydrolysis was not completely inhibited by TEPP, the influence of the increased Ch concentration must be taken into account in the evaluation of the above results. Therefore, the hydrolysis of 10 mM labeled ACh was determined in parallel experiments using paper chromatography. The results show that about 10 per cent of the radioactive ACh was hydrolysed at the end of the incubation period. Since about 2 per cent of the radioactivity was in the form of Ch already before the addition of the unlabeled ACh, probably less than 10 per cent of ACh was hydrolysed during incubation. In parallel experiments in which hydrolysis of unlabeled ACh was measured using Hestrin's method[16] only about 9 per cent of ACh was hydrolysed within 1 hr. As the Ch concentration at the end of the experiment had not been above 1 mM, the mean concentration during the experiment was estimated to have been not above 0·5 mM. In the experiments for the study of a possible mutual inhibition the ratio a_i/a_e obtained after 1 hr of incubation was about 1·30 (Fig. 4), whereas in the experiments in which the entry of Ch at a concentration of 0·5 mM was studied, the ratio a_i/a_e was 2·62 (Fig. 3). It seems that only part of the effect produced by 10 mM ACh on the entry of labeled Ch can be attributed to Ch formed by ACh hydrolysis, whereas the rest of the effect is probably due to ACh itself. Thus, ACh and Ch seem to inhibit each other.

In another set of experiments it was attempted to find out whether there was any difference between the ACh entry into the end-plate region and the ACh entry into the rest of the muscle. It was found that labeled decamethonium concentrates in the nerve ending region of the rat diaphragm, whereas labeled carbachol does not.[8] Even though in our experiments the concentration of radioactive ACh varied from 5 μM to 10 mM, no significant difference between the radioactivity per gram tissue of the end-plate region and that of the rest of the muscle, was observed. Nor was there any difference in the experiments without TEPP. Our results, however, do not exclude the possibility that ACh concentrates in the end-plate. The portion occupied by the end-plates and particularly by the nerve endings is very small in comparison with the overall volume of the muscle fibre. The possible higher rate of the ACh entry into these structures might be masked by the ACh entry into the rest of the muscle.

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