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EFFECT OF EXTERNAL pH ON THE CHOLINE ENTRY INTO THE RAT DIAPHRAGM MUSCLE FIBRE

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Abstract—The dependence of the entry of choline *N*-methyl-^{14}C into the rat diaphragm muscle fibre on the pH of the incubating solution was studied. At 20 mM choline concentration, when primarily the non-saturable component was measured, the choline entry did not change with a change in pH from 6·0 to 7·2. By increasing the pH to 9·0 the choline entry increased by about 40 per cent. At 5 μM choline concentration, when primarily the saturable entry component was measured, the choline entry increased with the increasing pH. The pH dependence of the choline entry was greater at lower pH values and diminished at higher ones. This effect of pH on the saturable component of the choline entry was found to be reversible. Kinetic study of the choline entry showed that by increasing the pH from 6·0 to 8·0 the K_m was decreased, whereas the maximum entry rate was not affected significantly. The results suggest that the passive component of the choline entry is only slightly affected by pH, whereas the carrier-mediated entry is considerably dependent on pH. The hydrogen ion might affect the choline entry by inhibiting the binding of choline to the choline carrier.

IT HAS been shown that the entry of choline into the rat diaphragm muscle fibre is mediated by a carrier,[1,2] and that this process is inhibited by acetylcholine[3] as well as by some quaternary ammonium compounds particularly those known as neuromuscular blocking agents.[4] This indicates a partial similarity between the choline carrier on the one hand and the cholinoreceptor and cholinesterase on the other. There is not enough data on the movement of choline across the cell membrane to explain the molecular basis of the choline entry mechanism. Therefore, further studies on the choline movement across the cell membrane under different experimental conditions are needed in order to obtain relevant data on the choline carrier.

Like the enzymatic processes, the carrier-mediated choline entry may be dependent on pH as has already been shown for the choline entry into the guinea-pig brain synaptosomes.[5] The effect of pH on the choline entry into the skeletal muscle fibre has not been studied. The aim of the present work was, therefore, to find out whether the entry of choline into the rat diaphragm muscle fibre is dependent on the pH of the incubating medium and if so to investigate the manner in which the external pH affects the entry kinetics.

METHODS

The experiments were carried out on isolated hemidiaphragms from albino rats of both sexes, weighing 100–150 g. The diaphragm was excised under ether anaesthesia and placed at room temperature in Krebs bicarbonate buffer[6] with 200 mg of glucose per 100 ml of buffer pH 7·4. The dorsal part of the diaphragm, 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 the central tendon was preincubated for 5 min at 38° in 10 ml of a buffer solution without choline. The pH values of the buffer solution ranged from 6·0 to 9·0 in intervals of 0·6 pH units. At the end of the preincubation period the pH of the solution was checked and the hemidiaphragm transferred to 10 ml of the incubating medium consisting of labelled and unlabelled choline dissolved in the same buffer solution with the same pH as used for the preincubation. Before and after incubation the pH of the incubating medium was checked. After 30 min of incubation at 38°, 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 measurements. The water content was determined by drying the muscle to constant weight. The mannitol extracellular space was determined by using labelled mannitol.

Since in our experiments the influence of pH within the range 6·0–9·0 was studied, buffers generally used in experiments with isolated tissue could not be employed. Therefore incubating solution containing Britton–Robinson buffer[7] was used in most of our experiments. The solution was composed of: NaCl, 120 mM; KCl, 5 mM; $MgSO_4$, 1·2 mM; acetic acid, 8·0 mM; boric acid, 8·0 mM; phosphoric acid, 8·0 mM; glucose, 5 mM; and NaOH from 18 mM (pH 6·0) to 27 mM (pH 9·0). The incubating solution was bubbled with oxygen. In some experiments solutions containing 20 mM phosphate buffer were used instead of the Britton–Robinson buffer, leaving the concentrations of NaCl, KCl, $MgSO_4$ and glucose unchanged. Although the buffer capacity was considerable, the pH value of each incubating solution changed during the incubation period. The pH shift was greater at higher pH values and after 30 min of incubation at pH 9·0 was about 0·2 pH units. In view of these pH shifts, the pH of each solution was adjusted in such a way that the calculated average of the pH value for the incubation period did not differ more than 0·05 pH units from the desired one.

Choline (*N*-methyl-^{14}C) with the specific radioactivity of 60 mCi/mmole, purchased from the Radiochemical Centre, Amersham, was kept 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, v/v).

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-phenyloxasolyl)] benzene (POPOP) and 80 g of naphthalene per litre of solvent consisting of equal volumes of toluene, *p*-dioxane and ethyleneglycol monomethyl ether.

RESULTS AND DISCUSSION

The influence of pH on the mannitol space of the diaphragm muscle was measured, since for choline entry calculations data on the extracellular space are necessary. Figure 1 shows that the mannitol space of the muscle incubated in solutions with Britton–Robinson buffer did not change appreciably when pH was increased from 6·0 to 7·2 and that it was only slightly decreased when pH was further increased to

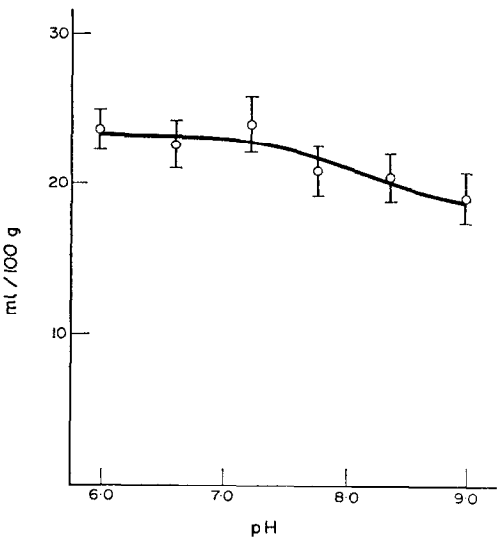

FIG. 1. The effect of pH on the mannitol-^{3}H space of the rat diaphragm. After 5 min of preincubation the hemidiaphragms were incubated for 30 min at 38° in a solution with Britton–Robinson buffer to which mannitol-^{3}H was added. The points give the mean of eight values with S.E.M. of the mean.

9·0. The mannitol space of the diaphragm muscle incubated in solutions with phosphate buffer was found to be only slightly larger than when the muscle was incubated in solutions with Britton–Robinson buffer and did not change when the pH ranged from 6·0 to 8·0. It seems that the extracellular space does not depend appreciably on the pH within the range tested. Using another buffer solution, Rorive and Kleinzeller[8] showed also that the inulin space of the rat diaphragm was unaffected by the pH within the range 6·2–8·2.

In our experiments solutions without Ca ions were used. In order to check whether the absence of Ca ions in the incubating solution affected the choline entry mechanism, choline entry into the diaphragm muscle fibre incubated in Krebs bicarbonate buffer, pH 7·4, with 1·2 mM Ca ions as well as without Ca ions, was measured. After 30 min of incubation the ratio between the concentration of labelled choline in the intracellular water and the concentration of labelled choline in the incubating medium with and without Ca ions was found to be 6·8 ($\pm$0·89) and 7·6 ($\pm$1·48), respectively. These figures, which are in good agreement with those obtained by Diamond and Kennedy[5] studying the influence of Ca ions on the choline entry into guinea-pig brain synaptosomes, suggest that there is no significant influence of Ca ions on the saturable component of the choline entry.

In order to study the dependence of the passive component of the choline entry on pH, the entry of choline at 20 mM concentration was measured in the pH range of 6·0 to 9·0. At this concentration of the substrate the non-saturable component of the choline entry is considerably larger than the saturable one.[9] The results presented in Fig. 2 show that the choline entry did not change when pH was increased from 6·0 to 7·2 and that by further increasing pH to 9·0 the choline entry was increased by about 40 per cent. It seems that the permeability of the muscle fibre membrane to monovalent cations is generally only slightly affected by the pH of the incubating solution.

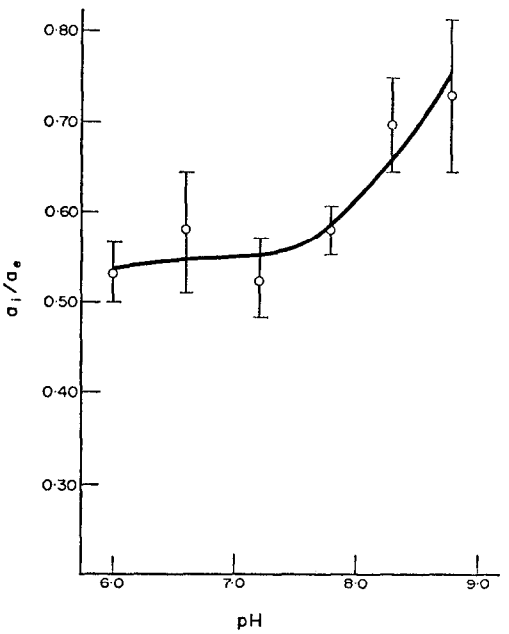

FIG. 2. The effect of the external pH on the non-saturable component of the choline entry into the rat diaphragm muscle fibre. After 5 min of preincubation the hemidiaphragms were incubated for 30 min at 38° in a solution with Britton–Robinson buffer to which choline-^{14}C and non-radioactive choline in 20 mM final concentration were added. The points give the mean of six values with S.E.M. of the mean.

It has been found that the potassium permeability of the barnacle muscle fibre is only slightly increased when pH is increased from 5·0 to 9·0,[10] that potassium permeability of the frog sartorius muscle is only slightly increased in alkaline solution,[11] and that the sodium concentration in the rat diaphragm muscle fibre is not affected by the pH of the incubating medium in the range pH 6·2 to 8·2.[8] The pH dependence of the non-saturable component of the choline entry into the muscle fibre, as determined in our experiments, seems to be similar to the pH dependence of the permeability of the muscle fibre membrane to sodium and potassium.

The influence of pH on the saturable component of the choline entry was studied at 5 μM substrate concentration. At that concentration the non-saturable component represents less than 5 per cent of the total choline entry.[9] The results obtained with diaphragms incubated in solutions with Britton–Robinson buffer, pH from 6·0 to 9·0, are shown in Fig. 3. The choline entry rate increased with the increasing pH. This dependence of the choline entry rate was greater at lower pH values and diminished with the increasing pH. By changing the pH from 6·0 to 6·6, the choline entry was increased by about 24 per cent and by only about 2 per cent when pH was increased from 8·4 to 9·0. In the experiments when phosphate buffer pH 6·0–8·0 was used the choline entry was found to depend on pH in roughly the same way as when Britton–Robinson buffer was used. Another series of experiments was designed in order to find out whether the pH effect was reversible or not. The hemidiaphragms were preincubated for 30 min in solutions with Britton–Robinson buffer, pH 6·0–9·0. Subsequently the preparation was rinsed with and

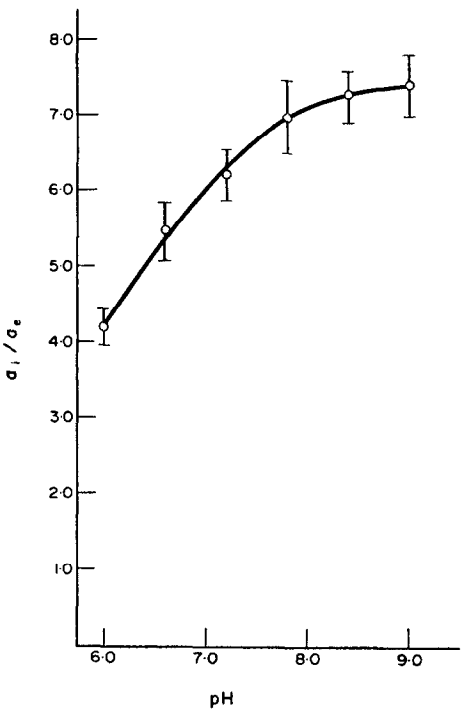

FIG. 3. The effect of the external pH on the saturable component of the choline entry into the rat diaphragm muscle fibre. After 5 min of preincubation the hemidiaphragms were incubated for 30 min at 38° in a solution with Britton–Robinson buffer to which choline-^{14}C in 5 μM final concentration was added. The points give the mean of nine values with S.E.M. of the mean.

incubated in a solution with Britton–Robinson buffer and labelled choline at pH 7·4. It was found that preincubation at different pH values did not affect significantly the choline entry at the physiological pH. The results suggest that in the range pH 6·0–9·0 the saturable component of the choline entry is reversibly affected by pH. The course of the pH dependence of the choline entry shown in Fig. 3 permits the assumption that at pH 9·0 the choline entry might attain its optimum value. When pH values higher than 9·0 were used, shifts of the external pH during the incubation of the hemidiaphragm increased so that a quantitative evaluation of the results was rather difficult. However, in some experiments when the external pH during the incubation shifted from 10·0 to 9·5 the choline entry was found to be less than it was at pH 9·0. A similar pH optimum for choline entry into the synaptosomes of the guinea-pig brain was found by Diamond and Kennedy.[5]

In another series of experiments the entry of choline at different substrate concentrations was studied in order to analyse the effect of pH on the entry kinetics. The rat hemidiaphragms were incubated in a medium at either pH 6·0 or 8·0. The results plotted according to Lineweaver–Burk as shown in Fig. 4 indicate that the K_m of the choline carrier which amounted to about 0·40 mM at pH 6·0 decreased to about 0·25 mM when the pH was increased to 8·0. On the other hand, it seems that the maximum entry velocity was not affected significantly by the same change in pH. These results,

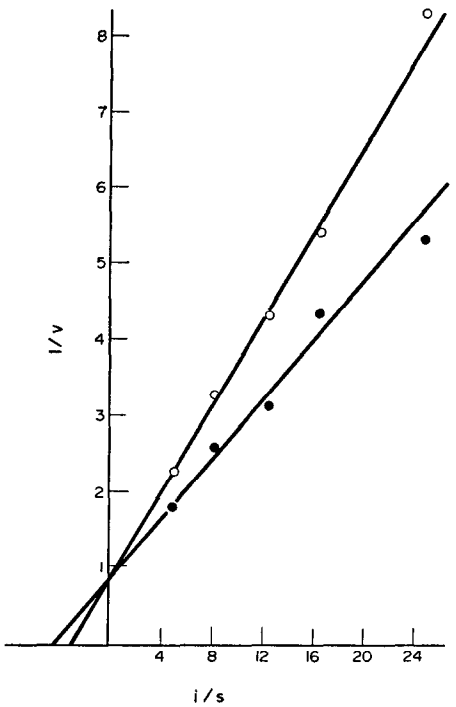

FIG. 4. The double reciprocal plot of the entry of choline at different substrate concentrations. After 5 min of preincubation the hemidiaphragms were incubated for 30 min at 38° in a solution with Britton–Robinson buffer to which choline-^{14}C in 5 μM final concentration was added. (●) External pH 8·0; (○) external pH 6·0.

therefore, suggest that the effect of pH on the choline entry is due mostly to the influence of pH on the affinity of the choline carrier. The effect of pH on the choline entry kinetics has not yet been studied. Data on other transport systems indicate that in various carrier-mediated processes the pH effect on the kinetics of the process might be rather different. It was found, for example, that in human erythrocytes only the maximal rate of the glucose exchange transport followed the changes in pH, whereas the K_m value remained constant;[12] on the other hand, in the active transport of potassium in yeast the K_m was found to be affected by pH.[13]

There are several possible levels at which the pH changes could affect the choline entry. It can be assumed that the effect of pH observed in our experiments was due to changes in the state of ionization of the components of the choline entry system. In this case the binding of the hydrogen ion to the hypothetical carrier would affect the binding of choline. If this is so, the effect is analogous to the competitive inhibition in enzymatic reactions. However, the state of ionization of other ionizable components of the membrane apart from the choline carrier might also change with the changing pH. In addition, it should be borne in mind that changes in the external pH might give rise to changes in the intracellular hydrogen ion concentration, even though an attempt was made to adjust our experimental conditions to minimize the changes in the intracellular pH.[14,15] It cannot be excluded that also the effects of the external pH referred to above might have influenced the results obtained in our experiments.

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