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Effect of cholinesterase inhibitors on active potassium influx in monkey erythrocytes

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The role of the acetylcholinesterase present in erythrocytes is still unknown. However, a number of reports in the literature have studied the possible involvement of acetylcholinesterase in ion transport and ion permeability in these cells.[1–4] In all these studies, uptake or release of sodium or potassium from erythrocytes were measured after prolonged incubation, i.e. for several hr or longer. In the following we report the effect of various cholinesterase inhibitors on active K^+-influx in monkey erythrocytes. The technique utilized permitted influx determination at short times (5–30 min) after exposure of the erythrocytes to the reagent tested.[5]

The compounds examined included three inhibitors of cholinesterase which inhibit the enzyme by covalently blocking the serine at the active site, diisopropylfluorophosphate, eserine and neostigmine, the substrate acetylcholine and two quaternary ammonium compounds, decamethonium and butyrylcholine, which can bind to the enzyme reversibly.

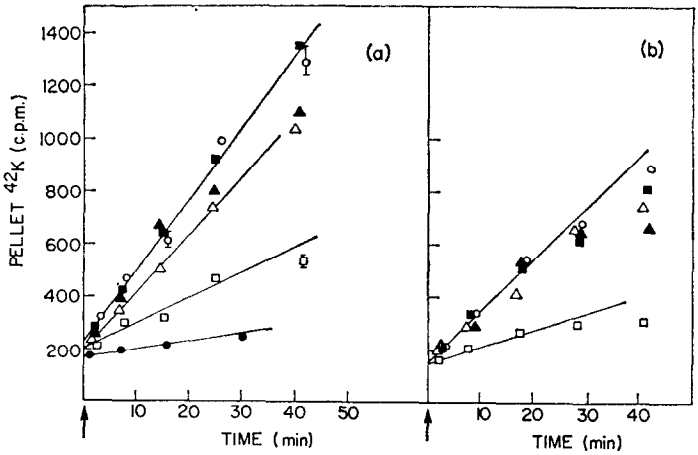

Fig. 1. Kinetics of uptake of ^{42}K by treated and control monkey erythrocytes. Samples were removed and the erythrocyte pellet separated and counted as described in the text. (a) ○, control; □, 0·1 mM eserine; △, 1 mM diisopropylfluorophosphate; ■, 0·075 mM neostigmine; ▲, 1 mM decamethonium; ●, 1 mM ouabain. (b) ○, control; □, 1·2 mM eserine; △, 1·2 mM diisopropylfluorophosphate; ■, 1·2 mM butyrylcholine; ▲, 27 mM acetylcholine. ↑, at zero time ^{42}K was added to the suspension.

In the experiments described below, Rhesus monkey erythrocytes from freshly drawn blood were washed twice and resuspended (about 9% haematocrit) in 4 mM KCl–136 mM sodium phosphate pH 7·3. Aliquots of 0·9 ml were then incubated in 13 mm diameter tubes at 37° with gentle shaking. After 2 min, 50 μl of a solution containing the appropriate reagent was added to each tube. Eight min later, 0·1 ml aliquots were withdrawn for cholinesterase activity determinations by the method of Ellman *et al.*[6] A 50 μl aliquot of a ^{42}K solution was then added to each tube. This solution contained 10 mM KCl–140 mM NaCl, pH 7·3 and contained about 400 μCi/ml.

The uptake of the radioisotope by the cells was obtained from radioactivity measurements of

erythrocyte pellets obtained at different time intervals. The cells were separated from the extracellular medium by the differential flotation method, as described previously.[5] Separations were carried out in polyethylene tubes of about 0·4 ml. Each tube contained 0·05 ml of the separating fluid (density of 1·066 g/cc) to which 0·1 ml of the erythrocyte suspension was added. The results of two typical experiments are shown in Figs. 1a and b. It can be seen that K^+-influx was linear over a period of 25 min, and that eserine consistently displayed a large inhibitory effect on K^+-influx, while diisopropylfluorophosphate had a relatively small effect. Although the degree of inhibition varied from experiment to experiment, the results shown in Fig. 1a are representative. The other compounds tested, neostigmine, acetylcholine, butyrylcholine and decamethonium had no significant effect. The results obtained in a number of experiments are tabulated in Table 1.

The enzymic activity of the rhesus monkey erythrocytes on acetylthiocholine was found to be in the range of $0{\cdot}5\text{–}1{\cdot}0 \times 10^{-9}$ μ moles per min per erythrocyte, in fair agreement with acetylcholinesterase activity values previously reported for monkey erythrocytes.[7] As would be expected at the concentrations employed, diisopropylfluorophosphate, eserine and neostigmine all caused complete inhibition

TABLE 1. EFFECT ON ACTIVE K^+-INFLUX OF IRREVERSIBLE ACETYLCHOLINESTERASE INHIBITORS, ACETYLCHOLINE ANALOGS AND OUABAIN

Reagent	Concentration (mM)	Inhibition of K^+-influx (%)
Eserine	0·9	27 ± 3
	1·2	70 ± 5
	0·1	66 ± 7
	0·1	27 ± 3
	0·3	41 ± 4
	0·3	33 ± 4
	0·13	40 ± 4
Diisopropylfluorophosphate	1·2	16 ± 2
	1·0	20 ± 2
	1·0	10 ± 2
Acetylcholine	3·8	8 ± 2
	27	0
Decamethonium	1·0	0
	1·0	15 ± 2
Butyrylcholine	1·2	0
	1·0	0
Neostigmine	0·08	0
	0·01	0
Ouabain	1·0	90
Ouabain + eserine	1·0; 0·1	90

of enzymic activity after the specified preincubation period. The quarternary ammonium compounds had no significant effect, but were, of course, greatly diluted in the final enzyme assay medium, as compared to their concentration in the K^+-influx experiments.

The results obtained, showing that complete inhibition of acetylcholinesterase by neostigmine and DFP had little or no effect on active K^+-influx, indicate that the enzyme is not directly involved in K^+-transport. The lack of effect of decamethonium and butyrylcholine, at the high concentrations at which they were employed, also supports this view. No explanation for the inhibitory action of eserine can at present be advanced. It is possible that at the relatively high concentrations employed some other system was affected. However, it is of interest that in two types of cells, baby hamster kidney cells (BHK 21) and *Mycoplasma laidlawii*, which did not display significant levels of acetylcholinesterase activity when assayed under the same conditions as the monkey erythrocytes, no inhibition of active K^+-influx by eserine was observed.

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Effect of α-tocopherol on passive monovalent cation uptake by rat liver mitochondria

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THE BIOCHEMICAL function of α-tocopherol in cell metabolism is not known. As this compound was found in mitochondria, its participation in the mitochondrial electron transport system either as a cofactor or as a structural agent has been suggested.[1–3] Mollenar *et al.*[4] suggested that α-tocopherol plays a decisive role in membrane metabolism. Oliveira *et al.*[5] found that α-tocopherol is present in the inner mitochondrial membrane and totally absent in the outer membrane. It is generally accepted that the inner mitochondrial membrane takes part a key regulatory role in anion and cation transport. These data led us to examine the effect of α-tocopherol on the transport of monovalent cations in mitochondria. The permeability of the mitochondrial membrane to various solutes has been investigated by a number of different techniques. In this work we used the osmotic swelling method of Chappell and Crofts,[6] who took advantage of the osmotic response of inner membrane to test the permeability of the mitochondrion to cations. It is well known that isolated heart and liver mitochondria swell rapidly when suspended in isotonic NH_4–acetate or Na–acetate in the absence of a source of energy.[7,8] On the other hand isolated mitochondria suspended under this condition in the corresponding acetate salts of K^+ or Li^+ swell very slowly.[8] None of the cations Na, K, Li support swelling as the Cl^- salts either in the presence or in the absence of a source of energy at neutral pH.[9] In this short report the effect of α-tocopherol on swelling of rat liver mitochondria suspended in acetate Na, K, Li, Rb or in corresponding Cl^- salts in the absence of a source of energy has been studied.

It was found that addition of α-tocopherol to rat liver mitochondria suspended in isotonic K^+ acetate (Fig. 1) or Rb^+ acetate (Fig. 2) resulted in a decrease of absorbance. The effect of α-tocopherol was compared on these figures with gramicidin. When α-tocopherol was added to the mitochondria suspended in isotonic KCl or isotonic RbCl the decrease of absorbance was less pronounced (not shown here). As may be seen on the figures the effect of α-tocopherol on mitochondrial swelling in K^+ or Rb^+ acetate media is similar to gramicidin, however less pronounced. Brierley[8] reported that gramicidin was without effect on mitochondrial swelling in isotonic KCl, and caused swelling in K^+ acetate media in the absence of an energy source. Similar effect we observed with α-tocopherol. It seems that in our conditions α-tocopherol increases the permeability of rat liver mitochondrial membrane to K^+ and Rb^+ in the absence of source of energy. The effect of α-tocopherol on permeability of mitochondrial membrane in the absence of energy seems to be specific for K^+ and Rb^+; when mitochondria were suspended in Li^+ acetate, α-tocopherol was without effect, when suspended in Na^+ acetate even a slight inhibition was observed (not shown here).

A number of different chemical modifications of the mitochondrial membrane have been shown to result in an increase of the energy-dependent or non energy-dependent ion uptake. The modifications include addition of an ionophore or ion carrier such as gramicidin or valinomycin,[10] addition of Zn^{2+},[8] organic mercurials and other thiol-group reagents,[11] nonionic detergent[12] or addition of parathyroid hormone.[13] The presence of α-tocopherol may be added to the list as it activates the non energy-dependent K^+ and Rb^+ uptake. It seems that this effect is dependent on the chemical interaction of α-tocopherol with the molecular arrangement of mitochondrial membrane. It may be important for regulation of permeability of mitochondrial membrane. A more complete account of this study will be presented later.