BBA 66323

A K$^+$-ACTIVATED, ETHACRYNIC ACID-SENSITIVE *p*-NITROPHENYLPHOSPHATASE FROM NORMAL HUMAN WHITE CELLS

G. J. S. RAO, M. DEL MONTE AND H. L. NADLER

Division of Genetics, Children's Memorial Hospital, Chicago, Ill., and Department of Pediatrics, Northwestern University School of Medicine, Chicago, Ill. (U.S.A.)

(Received December 8th, 1970)

SUMMARY

1. The nuclear fraction of normal human white cells contains a K$^+$-activated *p*-nitrophenylphosphatase.

2. This enzyme is optimally activated by 5 mM KCl, has a pH optimum of 7.0 and does not hydrolyse ATP, AMP, UTP, phosphoenolpyruvate or sodium β-glycerophosphate.

3. The enzyme is insensitive to ouabain but is inhibited by low concentrations of ethacrynic acid.

4. These findings suggest that it is a phosphatase different from those previously described.

Na$^+$–K$^+$-activated, ouabain-inhibited ATPases involved in the transport of Na$^+$ and K$^+$ across membranes have been studied in several tissues[1–5]. Present evidence suggests that the (Na$^+$–K$^+$)-ATPase complex contains a K$^+$-activated, ouabain-inhibited phosphatase[6–9]. (Na$^+$–K$^+$)-ATPases are also inhibited by ethacrynic acid, [2,3-dichloro-4-(2-methylene butyryl)phenoxy]-acetic acid and *p*-hydroxymercuribenzoic acid but not other thiol reagents such as iodoacetamide or *N*-ethylmaleimide[10].

Utilizing normal human white blood cells, we have obtained a preparation from the nuclear pellet containing an enzyme which hydrolyzes *p*-nitrophenyl phosphate. This enzyme is activated by K$^+$ and is inhibited by ethacrynic acid but not by ouabain. These properties suggest that the enzyme is distinct from the K$^+$-activated *p*-nitrophenylphosphatases previously reported.

Blood (100 ml) was collected from normal donors in heparinized syringes (2000 U.S.P. units) and mixed with 20 ml dextran. The red cells were allowed to sediment by gravity at 37°. The white cells in the plasma were separated according to NADLER AND HSIA[11], collected by centrifugation, homogenized in a Dounce apparatus (100 strokes) with 4.0 ml cold sucrose and the homogenate was centrifuged at 600 × *g*. The pellet was re-homogenized with 8.0 ml sucrose and centrifuged again. The pellet was washed three times with sucrose in this manner and homogenized in 8.0 sucrose to yield a uniform suspension. The ratio of K$^+$-dependent activity to

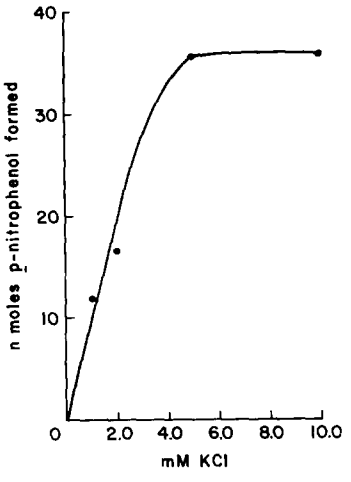

Fig. 1. Effect of KCl on the *p*-nitrophenylphosphatase activity. Ordinate represents increment in activity in the presence of K^+ over that in its absence. The incubation mixture was essentially similar to that of BONTING AND CARAVAGGIO[12] except that *p*-nitrophenyl phosphate (sodium salt) replaced ATP and cyanide was omitted. 0.5 ml of the reaction mixture contained 0.092 mM Tris HCl buffer (pH 7.0), 0.1 mM EDTA, 1 mM $MgCl_2$ and white blood cell extract corresponding to 87 μg protein. The reaction was initiated by the addition of *p*-nitrophenyl phosphate to a final concentration of 5 mM. The reaction mixture was incubated for 30 min by shaking in a 37° water bath and was terminated by adding 1.5 ml of 0.25 M NaOH. The absorbance of *p*-nitrophenol liberated was read at 410 nM. Appropriate blanks were run to correct for the non-enzymic breakdown of the substrate.

K^+-independent activity in the washed preparation was enhanced 2.6-fold relative to the crude pellet.

KCl activated the enzyme optimally between 5 and 10 mM (Fig. 1). The K^+-dependent activity was linear between 5 and 60 min and between 0.01 and 0.1 mg/ml of protein[13]. It was optimally active between pH 6.8 and 7.0 when assayed in 0.1 M Tris HCl or 0.1 M glycylglycine buffers. The preparation did not liberate inorganic phosphate[14] when sodium salts of AMP, ATP, UTP, phosphoenolpyruvate or β-glycerolphosphate were used as substrates either in the presence of 58 mM NaCl or

TABLE I

EFFECT OF ETHACRYNIC ACID AND THIOL REAGENTS ON THE ACTIVITY OF THE ENZYME

Assay conditions similar to that described under Fig. 1. Enzyme activity expressed as nmoles *p*-nitrophenol formed in 30 min.

Reagent (0.2 mM)	*Activity in the presence of* Mg^{2+}	*% Activity*	*Activity in the presence of* Mg^{2+} + K^+	*Difference activity due to* K^+	*% Activity*
None	12.7	100	52.0	39.3	100
Ethacrynic acid	11.4	80	12.0	0.6	1.8
N-Ethylmaleimide	1.9	15.1	9.6	7.7	19.4
p-Hydroxymercuri-benzoic acid	0.0	0.0	0.0	0.0	0.0
Iodoacetamide	11.0	87.0	42.0	31.0	78.5

5 mM KCl or a mixture of both. Ouabain (0.01–0.5 mM) had no effect on the activity either in the presence or absence of 5 mM KCl.

Ethacrynic acid (gift from Merck, Sharp, Dohme and Co.) completely inhibited the K^+-dependent activity without affecting the K^+-independent component (Table I). *p*-Hydroxymercuribenzoic acid completely inactivated both components and *N*-ethylmaleimide inactivated both components to the same extent although to a lesser extent than *p*-hydroxymercuribenzoic acid. Iodoacetamide was the least effective inhibitor.

Preincubation of the enzyme with ethacrynic acid[10] for 30 min at 37° eliminated the K^+-dependent activity retaining about 50% of K^+-independent activity (Fig. 2).

The K^+-activated *p*-nitrophenylphosphatase present in our preparations appears to be different from the *p*-nitrophenylphosphatases previously reported. This enzyme hydrolyzes only *p*-nitrophenyl phosphate, among the phosphate esters tested, and is insensitive to ouabain at concentrations as high as 0.5 mM. It is also distinct from the K^+-independent component of *p*-nitrophenyl phosphate because

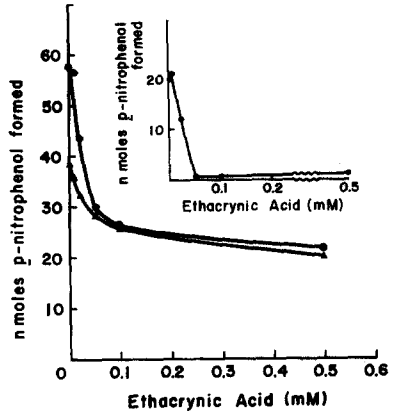

Fig. 2. Effect of ethacrynic acid in the presence and absence of KCl. KCl concentration in this and subsequent experiments was 5 mM. Ethacrynic acid (as Tris salt) was added to the incubation mixture containing white cell extract corresponding to 24 μg protein. After incubation for 30 min, *p*-nitrophenylphosphate was added to initiate the reaction. ●, activity in the presence of K^+ and Mg^{2+}; ▲, activity in the presence of Mg^{2+} alone. Inset: difference activity due to K^+.

the K^+-activated component can be inactivated by low concentrations of ethacrynic acid (0.05 mM) while retaining more than 50% of K^+-dependent component. The inhibition by ethacrynic acid may not be merely due to the reaction of ethacrynic acid with the thiol groups of the enzyme because a similar degree of inhibition was not observed with *N*-ethylmaleimide and iodoacetamide (Table I). In this respect, the inhibition by ethacrynic acid is similar to the inhibition of (Na^+–K^+)-ATPase by this compound[10].

The lack of inhibition by ouabain and the inability of the preparation to hydrolyze ATP, UTP, phosphoenolpyruvate and β-glycerolphosphate in the presence or absence of KCl, NaCl or a mixture of both suggests that the enzyme studied by us is a different enzyme with an, as yet, undiscovered natural substrate. However, its inhibition by ethacrynic acid, similar to (Na^+–K^+)-ATPase indicates that it may

be a component of (Na^+–K^+)-ATPase which is either inactive under present conditions of incubation or is inactivated during the preparation.

ACKNOWLEDGEMENTS

We wish to express our thanks to Drs. Ira Salafsky and Stephen Wood for their comments on the manuscript and to Mrs. Ruth Nyberg for her technical assistance. This investigation was aided by grants from the U.S. Public Health Service HD00036, The Chicago Community Trust and The Irene Heinz and John Laporte Given Foundation.

REFERENCES

1 R. L. Post, C. R. Merrit, C. R. Kingsolving and C. D. Albright, *J. Biol. Chem.*, 235 (1960) 1796.
2 E. T. Dunham and I. M. Glynn, *J. Physiol.*, 156 (1961) .
3 J. C. Skou, *Biochim. Biophys. Acta*, 23 (1957) 394; 42 (1960) 6.
4 R. Whittam and K. P. Wheeler, *Biochim. Biophys. Acta*, 51 (1961) 622.
5 J. C. Skou, *Physiol. Rev.*, 45 (1965) 596.
6 J. D. Judah, K. Ahmed and A. G. M. McLean, *Biochim. Biophys. Acta*, 65 (1962) 472.
7 P. Emmelot and C. J. Bos, *Biochim. Biophys. Acta*, 121 (1966) 375.
8 H. Yoshida, F. Izumi and K. Nagai, *Biochim. Biophys. Acta*, 120 (1966) 183.
9 H. Bader and A. K. Sen, *Biochim. Biophys. Acta*, 118 (1966) 116.
10 D. E. Duggan and R. M. Noll, *Arch. Biochem. Biophys.*, 109 (1965) 388.
11 H. L. Nadler and D. Y. Y. Hsia, *Clin. Biochem.*, 1 (1968) 192.
12 S. L. Bonting and L. L. Caravaggio, *Arch. Biochem. Biophys.*, 101 (1963) 37.
13 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1957) 265.
14 C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, 66 (1925) 375.