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DIRECT STIMULATION OF HUMAN ERYTHROCYTE MEMBRANE ($Na^+ + K^+$)-Mg ATPase ACTIVITY IN VITRO BY PHYSIOLOGICAL CONCENTRATIONS OF d-ALDOSTERONE

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SUMMARY: The effect of d-aldosterone on human erythrocyte ghost ($Na^+ + K^+$)-Mg ATPase has been studied. Aldosterone at 3.225×10^{-10}M caused a 450% activation of ($Na^+ + K^+$)-Mg ATPase activity whilst inhibiting ($Na^+ + Na^+$)-Mg ATPase activity. Aldosterone acts by reducing the affinity of the external K^+ site of ($Na^+ + K^+$)-Mg ATPase for Na^+ thereby resulting in improved efficiency of $Na^+ - K^+$ transfer. Aldosterone was additionally found to modify both the Na^+ and K^+ activation of ($Na^+ + K^+$)-Mg ATPase incubated in the presence of commercial ATP containing orthovanadate. Aldosterone was found to reverse the inhibitory effects of orthovanadate at high Na^+ and K^+ concentrations. The physiological significance of orthovanadate and aldosterone are discussed.

INTRODUCTION The ($Na^+ + K^+$)-Mg ATPase (E.C. 3.6.1.3) isolated from a variety of tissues is known to possess a steroid-binding site and to be inhibitable by a number of cardiotonic steroids; notably ouabain (1,2). The physiological significance of this site has remained obscure despite conjectural investigations with the mineralocorticoid aldosterone on membrane and purified enzyme preparations, where no direct effects were observed (3-8); although the concentrations of aldosterone employed were four orders greater than the normal maximum "free" concentration in serum of 2.2×10^{-10}M.

Examination of urinary Na/K ratios following administration of aldosterone to rats and dogs, reveals a progressive decrease of the ratio from the time of injection (9,10), there being no apparent latent period. This is suggestive of a direct sodium pump stimulation by aldosterone and consequently an investigation using physiological concentrations of aldosterone on erythrocyte ghost ($Na^+ + K^+$)-Mg ATPase activity was undertaken.

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MATERIALS AND METHODS

1. Preparation of Ghosts: Red cells from fresh human heparinized venous blood were washed three times with nine volumes of 0.15 M choline chloride (pH 7.4). Following the first wash, white cells and buffy coat were removed by aspiration. Unsealed ghosts were prepared by rapid introduction of 1 volume of washed red cells at 50% haematocrit into 15 volumes of swirling ice-cold NaH_2PO_4 (5.0mM pH 7.7). Membranes were centrifuged at 25,000g for 30 min. at 2°C and the supernatant removed and ghosts washed once in 10mM Tris-HCl (pH 7.7) at 4°C.

Following the final wash, three volumes of distilled water were added to the membranes and the suspension stored for 12 hr. at 4°C.

2. (Na + K)-Mg ATPase activity: Stoppered tubes containg 50mM NaCl, 10mM KCl, 2mM MgCl, 2.0mM 'Sigma Grade' ATP-Na_2, 250 µl ghosts, 100mM Tris-HCl (pH 7.4 at 37°C) were prepared at 4°C. Incubations lasting 2.5 hr. were initiated by warming tubes to 37°C after the addition of 5 ul aliquots of ethanol containing aldosterone or ouabain. Controls contained ethanol only. Incubations were terminated by cooling to 4°C and addition of ice-cold $HClO_4$ (0.5M, 0.5ml). Tubes were centrifuged at 4,000g (4 min.) after standing at 4°C for 20 min. Inorganic phosphorus released was estimated on the supernatant (11), and protein content estimated on the precipitate (12). Enzyme activity is expressed as µ mol Pi per milligram of membrane protein per hour (µmol $mg^{-1}hr^{-1}$).

The amount of ATP hydrolysed was less than 10% of the total in all cases and results are corrected for the non-enzymatic release of Pi from ATP and ghost membranes.

3. (Na^+ + Na^+)-Mg ATPase activity: Conditions as per section 2, except NaCl and KCl were omitted. Na^+ is provided by ATP-Na_2 'Sigma Grade'.

4. K^+ activation of (Na^+ + K^+)-Mg ATPase: Conditions as per section 2, except NaCl 10mM, ATP-Na_2 was replaced by ATP-Tris. K^+ was varied between 0-100mM according to legends of Fig. 3. Isotonicity was maintained with Tris.

5. Na^+ activation of (Na^+ + K^+)-Mg ATPase: Conditions as per section 2, except KCl 5mM, ATP-Tris replaced ATP-Na_2. Na^+ was varied between 0-100mM according to legends of Fig. 4. Isotonicity was maintained with Tris.

6. Mg-ATPase activity (E.C. 3.6.1.4.): Conditions as per section 2, except NaCl and KCl were omitted and ATP-Tris replaced ATP-Na_2.

RESULTS AND DISCUSSION

(Na^+ + K^+)-Mg ATPase activity. Aldosterone directly stimulates the sodium pump of unsealed erythrocyte ghosts (Fig. 1). The response characteristic is parabolic with a maximum activation at 3.225×10^{-10}M aldosterone, corresponding to the normal maximum physiological serum level. The magnitude of stimulation is 450% relative to controls. Assuming 150 sodium pumps/RBC (13) and a packed cell count before haemolysis of 9×10^9 RBC/ml, it is

calculated that there are two steroid molecules per $(Na^+ + K^+)$-Mg ATPase at this maximally activating concentration of aldosterone. The parabolic nature is suggestive of two binding sites, one high affinity which is stimulatory and another lower affinity site of an inhibitory nature. At 10^{-6}M, the activity obtained parallels control values and may explain the failure of previous investigators to demonstrate stimulatory properties, since concentrations in the range 10^{-6} were used (3-8). In order to demonstrate a specific activation by aldosterone, dehydroepiandrosterone, cholesterol and cortisol were similarly tested and found to be without effect.

The effect of aldosterone on $(Na^+ + Na^+)$-Mg ATPase activity. $(Na^+ + K^+)$-Mg ATPase has been shown to catalyse Na^+ - Na^+ exchange in K^+-free media. This activity is thought to occur as a consequence of Na^+ activating at the externally orientated K^+ site and is ouabain inhibitable (14). Aldosterone inhibits $(Na^+ + Na^+)$-Mg ATPase, virtually abolishing this activity at 3.225×10^{-10}M (Fig. 2). A statistical comparison of activities in Figs. 1 and 2 between the range 0 - 3.225×10^{-9}M aldosterone reveals a near perfect correlation ($r = -0.969$). This is indicative that aldosterone acts by inhibition of the Na^+ activation at the external K^+ site, thus the overall effect is to increase the efficiency of Na^+ - K^+ exchange and stimulate activity at high K^+ concentrations.

Interestingly Spach and Streeten (15) were able to demonstrate that 2.2×10^{-6}M aldosterone inhibited Na^+ - Na^+ exchange across the dog red cell membrane catalysed by $(Na^+ + K^+)$-Mg ATPase thus supporting the data presented here.

Mg ATPase activity. Aldosterone was found to be without effect on Mg-ATPase activity at all concentrations tested (0 - 10^{-6}M). Data not shown.

K^+ activation of (Na + K)-Mg ATPase. Following the demonstration of an aldosterone inhibition of $(Na^+ + Na^+)$-Mg ATPase activity and the formulation of a theory involving modified activation by K^+,

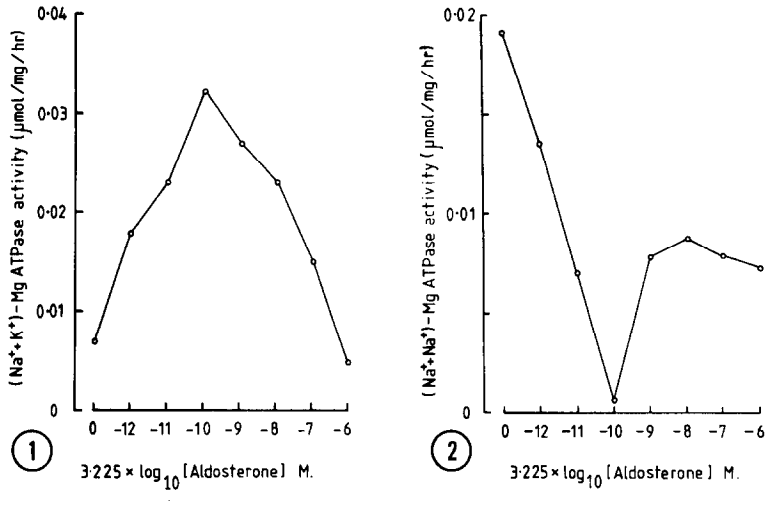

Fig. 1. The effect of d-aldosterone on red cell ghost $(Na^+ + K^+)$-Mg ATPase activity. Results are corrected for Mg-ATPase activity which was 0.0149 µmol mg^{-1} hr^{-1}.

Fig. 2. The effect of d-aldosterone on $(Na^+ + Na^+)$-Mg ATPase activity of erythrocyte ghost membranes. Corrected for Mg-ATPase activity.

investigation of the effect of 2.0×10^{-10}M adlosterone on the K^+ activation of $(Na^+ + K^+)$-Mg ATPase was performed. Examination of Fig. 3 shows that high levels of K^+ are inhibitory in the presence of 'Sigma grade' ATP (Sigma London Ltd.). This is not the normal response to K^+ and is due to the presence of orthovanadate in the ATP preparation (16). Aldosterone at 5×10^{-10}M reverses the inhibitory effects of high K^+ levels and leads to speculation about an interaction of the two compounds *in vivo*. Tests with synthetic ATP not containing orthovanadate show that the presence of vanadium is not a pre-requisite for the aldosterone effect, a stimulation of activity at high K^+ levels still occurs in the absence of orthovanadate in response to aldosterone. Vanadium is a dietary requirement for man (17,18) and is normally present in serum at a concentration of 1µM, a level known to cause a significant inhibition of $(Na^+ + K^+)$-Mg ATPase activity. The inhibitory effects of orthovanadate are also reversed by high concentrations of catecholamines

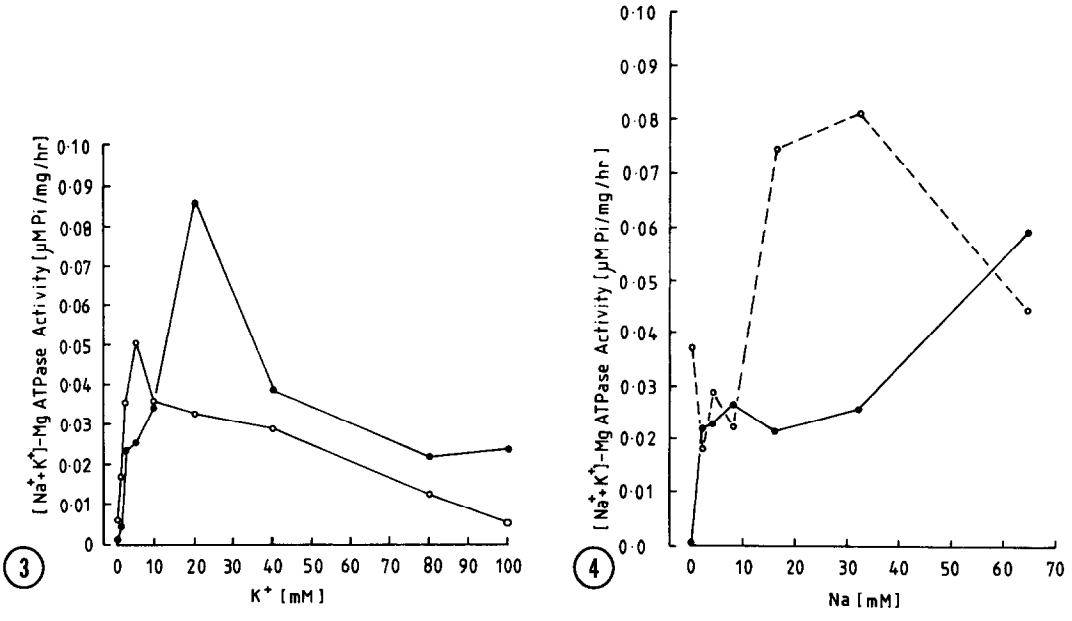

Fig. 3. The effect of d-aldosterone on the K^+ activation of $(Na^+ + K^+)$-Mg ATPase activity of erythrocyte ghost membranes incubated in the presence of ATP containing orthovanadate. Control(o), 5×10^{-10}M aldosterone(●). Na^+ 10 mM, corrected for Mg ATPase activity.

Fig. 4. The effect of d-aldosterone on the Na^+ induced activation of $(Na^+ + K^+)$-Mg ATPase from erythrocyte ghost membranes incubated in the presence of ATP containing orthovanadate. Control(●), 1×10^{-10} Aldosterone(o). K^+ 5mM, data corrected for Mg ATPase activity.

(16,19,20). It appears that aldosterone and possibly catecholamines may relieve inhibition by orthovanadate in vivo. The result of this at the kidney would be increased reabsorption of Na^+, consequently the level of endogenous vanadium may play an important role in the regulation of the sodium pump and therefore the excretion and retention of K^+ and Na^+ by the kidney. Excessive renal Na^+ retention is known to cause hypertension and interestingly, vanadium has been implicated in arteriosclerosis and hypertension (21) and administration lowers serum cholesterol levels in man (22).

Fig. 4 illustrates the effect of 1.0×10^{-10}M aldosterone on the Na^+ activation of $(Na^+ + K^+)$-Mg ATPase. The activation produced by

Na^+ is, similarly to K^+, not the normal response for this system and is due to the presence of orthovanadate. Aldosterone modifies the activation produced, the overall effect being one of activation in the intermediate Na^+ range. Interestingly at 0mM Na^+, aldosterone appears to stimulate a K^+ dependent phospatase activity which is not observed in the absence of aldosterone or K^+. As with the K^+ activation (Fig. 3), it is not possible to say whether external K^+ or Na^+ is responsible for inhibition at high concentrations as the ghosts are unsealed. However Beaugé, L.A. and Glynn, I.M. were able to demonstrate that external K^+ was responsible for inhibition at high K^+ levels (personal communication), however data obtained by us using intact erythrocytes with varying intracellular Na^+ and K^+ values clearly shows that aldosterone caused a loss of intracellular K^+ and gain of Na^+ when intracellular K^+ was 110mM, but not when K^+ was 90mM. Interestingly at 34mM internal K^+ a strong stimulation of K^+ uptake was observed (unpublished) thus supporting the data in Fig. 3. It thus appears that intra and extracellular Na^+ and K^+ modulate any response to aldosterone.

Examination of the specific activity of our preparations reveals them to be somewhat lower than others (19,23). This is due to a high level of membrane bound haemoglobin, which gives a false impression of activity since data are expressed as μmol/mg membrane protein/hr. The high level of membrane haemoglobin is a consequence of only one washing of the ghosts after haemolysis, as we have found that repeated washing (3 - 4 times) of the membranes renders them unresponsive to aldosterone.

The transcriptional action of aldosterone on target tissues by intervention in nuclear control of electrolyte metabolism is generally accepted as the *in vivo* mode of action for aldosterone (24,25). The present studies indicate in addition, a direct mode of action capable of explaining many deficiencies of the previous theories, and it is anticipated that research on the interaction of the two mechanisms will

provide a new understanding of the role of this steroid in electrolyte metabolism.

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REFERENCES

1. Albers, R., Koval, G. and Siegal, E. (1968) Molec. Pharmacol. 4, 324 - 326.
2. Hoffman, J.F. (1969) J. Gen. Physiol. 54, 343s - 353s.
3. Glynn, I.M. (1957) J. Physiol. (London) 136, 148 - 173.
4. Sulser, F. and Wilbrandt, W. (1957) Helv. Physiol. Acta. 15, C37 - C39.
5. Streeten, D.H.P. and Solomon, A.K. (1954) J. Gen. Physiol, 37, 643 - 644.
6. Hill, J.H., Cortas, N. and Walser, M. (1973) J. Clin. Invest., 52, 185 - 189.
7. Bonting, S.L. and Canady, R.M. (1964) Am. J. Physiol. 207, 1005 - 1009.
8. Chignell, C.F., Roddy, P.M. and Titus, E.O. (1965) Life Sci. 4, 559 - 566.
9. Morris, D.J., Berek, J.S. and Davis, R.P. (1973) Endocrinology. 92, 989 - 993.
10. Morris, D.J. and Davis, R.P. (1974) Metabolism. 23, 473 - 494.
11. Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375 - 400.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265 - 275.
13. Drickamer, L.K. (1975) J. Biol. Chem. 250, (5) 1952 - 1954.
14. Garrahan, P.J. and Glynn, I.M. (1967) J. Physiol. (London) 192, 189 - 216.
15. Spach, C. and Streeten, D.H.P. (1964) J. Clin. Invest. 43, (2), 217 - 227.
16. Josephson, L. and Cantley, L.C. (1977) Biochem. 16, 4572 - 4578
17. Schroeder, H.A., Balassa, J.J. and Tipton, I.H. (1963) J. Chron. Dis. 16, 1047 - 1071.
18. Dimond, E.G., Caravaca, J. and Beochimol, A. (1963) Am. J. Clin. Nutr. 12, 49 - 53.
19. Hudgins, P.M. and Bond, G.H. (1977) Biochem. Biophys. Res. Comm. 77, (3) 1024 - 1029.
20. Fagan, J.B. and Racker, E. (1977) Biochem. 16, (1) 152 - 158.
21. Badadzhanov, S.N. (1975) Kardiologia. 15, (6) 133 - 134.
22. Curran, G.L., Azarnoff, D.L. and Bolinger, R.E. (1959) J. Clin. Invest. 38, 1251 - 1261.
23. Chan, P.C., Calabrese, V. and Theil, L.S. (1964) Biochim. Biophys. Acta. 79, 424 - 426.
24. Sharp, G.W.G. and Leaf, A. (1966) Physiol. Rev. 46, 593 - 633.
25. Fimognari, G.M., Fanestil, D.D. and Edelman, I.S. (1967) Am. J. Physiol. 213, 954 - 962.