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ABSENCE OF EFFECT OF ALDOSTERONE ON SODIUM EFFLUX CATALYZED BY THE HUMAN ERYTHROCYTE Na^+, K^+-ATPase *IN VITRO*

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Summary—The effect of physiological and pharmacological concentrations of aldosterone on Na^+ efflux catalyzed by the human erythrocyte Na^+,K^+-ATPase *in vitro* were studied. Aldosterone had no significant effect on ouabain-sensitive Na^+ efflux from fresh erythrocytes. In addition, aldosterone did not alter Na^+ transport activity of stimulated Na^+,K^+-ATPase of Na^+ loaded erythrocytes. Finally, Na^+ efflux from Na^+ loaded erythrocytes was not changed by preincubation of the cells with aldosterone. It is concluded that aldosterone *in vitro* does not modify pump activity of the human erythrocyte Na^+, K^+-ATPase.

INTRODUCTION

It is currently believed that an increase in Na^+,K^+-ATPase activity of epithelial cells is not due to a direct action of aldosterone on this transport enzyme, but rather to stimulation of some other rate-limiting steps of Na^+ transport [1]. However, aldosterone has also been recently suggested to interact directly with the Na^+, K^+-ATPase of non-epithelial cells. Hamlyn and Duffy[2] showed that incubation of rat erythrocytes with physiological concentrations of aldosterone led to a marked stimulation of ouabain-sensitive ATP hydrolysis. This finding was confirmed by results from Stern *et al.* in human erythrocytes [3]. In contrast, other authors failed to demonstrate any effect of aldosterone *in vitro* on hydrolytic activity of the human erythrocyte Na^+,K^+-ATPase [4]. Furthermore, a direct effect of aldosterone on Na^+ transport activity of the enzyme was not demonstrated in human erythrocytes [5, 6]. However, these investigators used concentrations of aldosterone 3–4 times the physiological range and other methodological differences can not be excluded. Therefore, the present study was undertaken to further investigate the direct effect of physiological and pharmacological concentrations of aldosterone on Na^+ efflux catalyzed by the Na^+, K^+-ATPase of human erythrocytes under different experimental conditions *in vitro*.

MATERIALS AND METHODS

Preparation of erythrocytes

Venous blood (20–40 ml) collected in heparinized tubes, was centrifuged at 1750 ***g*** for 10 min at 4°C and the plasma and buffy coat were aspirated. Erythrocytes were used immediately.

Na^+ loading of erythrocytes

The erythrocytes were loaded with Na^+ using a slight modification of a procedure which has been described previously [7]. Erythrocytes were washed twice with cold 110 mmol/l $MgCl_2$ and suspended in a Na^+ medium to give a hematocrit of 4–5%. The Na^+ medium contained (mmol/l): NaCl 140, sodium phosphate buffer (pH 7.4 at 37°C) 2.5, $MgCl_2$ 1, inosine 3, glucose 10 and neutral EGTA (ethyl-eneglycol-bis (beta-amino-ethyl-ether)N,N'-tetra-acetic acid) 1. Neutral EGTA was prepared by titration with Trizma base [*Tris*(hydroxy-methyl) aminomethane] to pH 7.4 at 37°C. The osmolality of the loading solution was adjusted to 295 ± 5 mosm/kg. The cells were incubated in this Na^+ medium for 2 h at 37°C. The suspension medium was renewed once after 1 h incubation. At the end of the treatment the cells were spun down at 4°C for 10 min at 1750 ***g*** and the supernatant was discarded. The hemoglobin content per liter of treated cells was much the same as that from fresh cells, suggesting no change in cell volume secondary to Na^+ loading procedure.

Measurement of Na^+ efflux

Na^+ efflux was measured using previously published methods [8, 9]. Fresh and treated cells were washed 5 times with cold 110 mmol/l $MgCl_2$ and suspended to a hematocrit of 20–25% in Mg^{2+}–sucrose medium. The Mg^{2+}–sucrose medium contained (mmol/l): $MgCl_2$ 75, sucrose 85, MOPS [3-N-(morpholino)-propane sulfonic acid]–Tris (pH 7.4 at 37°C) 10 and glucose 10. Osmolality was maintained at 295 ± 5 mosm/kg. A portion (1 ml) of the cell suspension was added to two tubes containing 15 ml of cold Mg^{2+}–sucrose medium with (a) KCl 2 mmol/l (K^+ medium) and (b) ouabain 0.1 mmol/l (ouabain medium). The resulting suspensions were pipetted into 16 tubes (2 ml/tube) and incubated at 37°C with continuous agitation. Two tubes containing cells incubated in K^+ medium were removed at 10,30, and 45 min, and two tubes containing cells incubated in ouabain medium were removed at 30, 60, 90, 120, and 150 min. The tubes were transferred to 0°C for 1 min and then centrifuged at 4°C for 3 min at 1750 ***g***

and the supernatant was removed, avoiding pellet contamination. Na^+ content was measured by atomic absorption spectrophotometry.

Calculation of the Na^+ efflux catalyzed by the Na^+,K^+-ATPase

Ouabain-sensitive Na^+ efflux (V) as an index of Na^+ efflux catalyzed by the Na^+,K^+-ATPase was computed using the following equation:

$$V = \frac{(D_{cat}) \times (1\text{-final hematocrit})}{\text{final hematocrit} \times t},$$

where D_{cat} represents the difference in external Na^+ concentration between tubes with K^+ and tubes with ouabain after the incubation time t.

The effects of aldosterone

The effects of aldosterone on Na^+ efflux catalyzed by the Na^+,K^+-ATPase were studied using the protocol described above. In one set of experiments the efflux of Na^+ from fresh erythrocytes was measured with and without aldosterone in the efflux media. In other experiments the effect of aldosterone on Na^+ efflux was determined in cells which had been preincubated in Na^+ medium with and without aldosterone. Aldosterone was dissolved in a minimal amount of ethanol provided that the final concentration of this solvent had no effect *per se* on Na^+ efflux. Statistical analysis was made by the paired Student's t-test.

RESULTS

Studies in fresh cells

Ouabain-sensitive Na^+ efflux as a function of aldosterone concentration was measured in fresh erythrocytes from five different healthy blood donors.

Aldosterone did not alter significantly ouabain-sensitive Na^+ efflux from fresh erythrocytes (Fig. 1). A maximal stimulatory effect was observed at an aldosterone concentration of 10^{-9} mol/l, when the flux had increased to 1980 ± 636 from 1440 ± 377 μmol/l cells per h (mean ± SD); (Table 1).

Table 1. The effect of aldosterone on the ouabain-sensitive Na^+ efflux from human erythrocytes

Aldosterone concn (mol/l)	Ouabain-sensitive Na^+ efflux (μmol/l cells per h)	
	Fresh cells	Na^+ loaded cells
0	1440 ± 377	3490 ± 1347
10^{-12}	1530 ± 420	3172 ± 957
10^{-11}	1822 ± 751	3367 ± 1221
10^{-10}	1863 ± 802	3124 ± 1074
10^{-9}	1980 ± 636	3227 ± 1019
10^{-8}	1836 ± 477	3093 ± 937

Values are given as mean ± SD of five experiments for each group of cells.
Internal Na^+ content (mmol/l cells): fresh cells = 6.2 ± 1.2; Na^+ loaded cells = 19.5 ± 2.4.

Studies in Na^+ loaded cells not preincubated with aldosterone

It has been demonstrated that the increase in erythrocyte Na^+ content does stimulate Na^+ efflux catalyzed by the Na^+, K^+-ATPase [10]. Therefore, the stimulation of ouabain-sensitive Na^+ efflux by the increase in internal Na^+ was measured at different aldosterone concentrations.

Figure 1 shows that aldosterone did not modify significantly the activity of the Na^+,K^+-ATPase of Na^+ loaded erythrocytes. Aldosterone 10^{-8} mol/l maximally decreases by a 12% the control flux (3093 ± 937 and 3490 ± 1347 respectively, mean ± SD of five experiments) (Table 1). The aldosterone concentration for half-maximal inhibition (IC_{50}) was 10^{-9} mol/l.

Studies in Na^+ loaded cells preincubated with aldosterone

Since human erythrocytes contain aldosterone at concentrations similar to that in plasma [11], the ATPase in the erythrocytes may already be under the

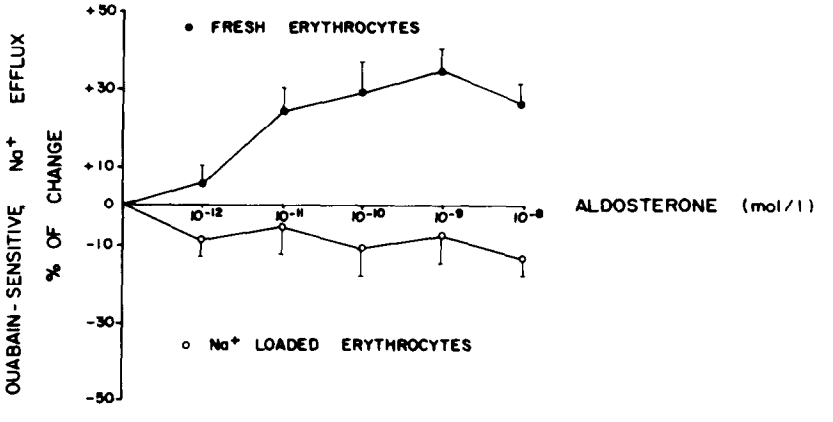

Fig. 1. The effect of aldosterone on Na^+ efflux catalyzed by the Na^+, K^+-ATPase of fresh (●, mean + SD) and Na^+ loaded (○, mean − SD) erythrocytes.

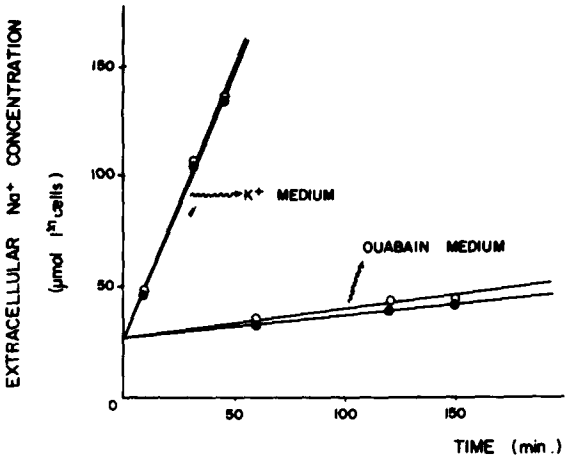

Fig. 2. Na^+ efflux from erythrocytes preincubated with (●) or without (○) aldosterone 10^{-8} mol/l.

influence of the hormone at basal conditions. We have therefore studied the effect of aldosterone 10^{-10}mol/l on Na^+ efflux from Na^+ loaded erythrocytes which previously had been incubated with aldosterone at a concentration of 10^{-8} mol/l.

As shown in Fig. 2, Na^+ efflux from cells preincubated with aldosterone was not different from Na^+ efflux from cells not preincubated with the hormone. Furthermore, the addition of aldosterone 10^{-10} mol/l to the efflux media did not change Na^+ efflux in preincubated cells as compared to control cells (Fig. 3).

DISCUSSION

These results indicate that aldosterone at physiological and pharmacological concentrations fails to affect directly pump activity of the fresh erythrocyte Na^+,K^+-ATPase. This confirms the findings of previous authors [3, 5, 6]. However, our results apparently contrast with those of authors who demonstrated that aldosterone at physiological concentrations increase hydrolitic activity of the enzyme *in vitro* [2, 3]. According to Stern *et al.*[3], one explanation of the unchanged Na^+ transport with an increased ATP hydrolysis would be that aldosterone increases the energy expenditure of the enzyme with no actual gain in its pumping activity. This hypothesis is supported by recent advances on the knowledge of reaction mechanisms of the Na^+,K^+-ATPase. In fact, the ATP–ADP exchange reaction and the associated transport of Na^+ ions are probably linked but are not tightly coupled, so that the enzymatic reaction can proceed in the absence of ion transport [12].

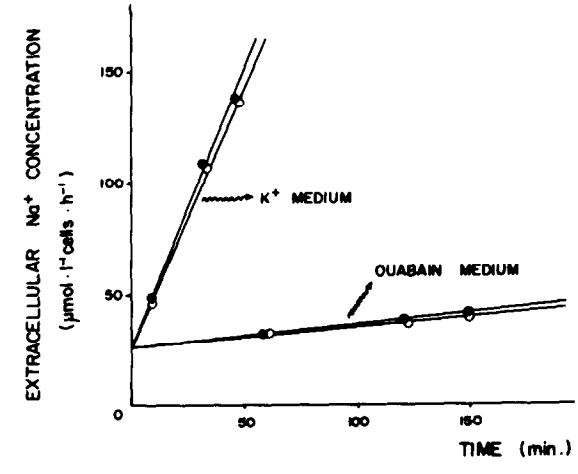

Fig. 3. The effect of aldosterone 10^{-10} mol/l on Na^+ efflux from erythrocytes preincubated with (●) or without (○) aldosterone 10^{-8} mol/l.

Aldosterone does not modify directly Na^+ transport activity of the erythrocyte Na^+,K^+-ATPase stimulated by the increase in internal Na^+. It has been shown that, as in the erythrocyte, activity of the kidney tubule Na^+,K^+-ATPase is stimulated by increase in cytoplastic Na^+ concentration [13]. Therefore, since aldosterone promotes the increase in Na^+ content of tubular target cells (by increasing Na^+ influx from the lumen), and this, in turn, may stimulate the activity of the baso-lateral Na^+,K^+-ATPase [14], our findings would suggest that in physiological conditions aldosterone does not interact directly with the Na^+-stimulated Na^+,K^+-ATPase of its renal target cells.

We have observed that preincubation in the presence of physiological concentrations of aldosterone does not modify subsequent Na^+ efflux from Na^+ loaded erythrocytes. Furthermore, ulterior incubation with aldosterone does not change Na^+ efflux from cells preincubated with the hormone. These results are not in agreement with those previously reported by Stern *et al.*[3] and Gall *et al.*[5], respectively. Our findings would suggest that the incorporation of aldosterone into the erythrocytes is not associated with a direct interaction of the hormone with the Na^+,K^+-ATPase on the inside of the membrane. This suggestion is in agreement with the fact that intracellular effects of aldosterone require the binding of the hormone to a cytoplasmatic receptor to form a complex which interacts with specific chromatin sites to initiate a cascade of events including RNA and protein synthesis [15]. Of course, it is not possible for this chain of events to occur in anucleated cells as the erythrocytes.

In summary, results presented in this paper indicate that pump activity of the human erythrocytes Na^+,K^+-ATPase is not modified by aldosterone directly *in vitro*.

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