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Research Article

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Glucocorticoid-induced Alterations in the Sodium Potassium Pump of the Human Erythrocyte

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ABSTRACT To evaluate the effects of glucocorticoids on the Na-K pump in human subjects, we evaluated the intracellular sodium and potassium, ^{42}K influx across and the [^3H]ouabain binding to cell membranes of intact human erythrocytes from a group of subjects taking glucocorticoids and a group of normal subjects. Intracellular sodium concentration was lower (7.2 ± 0.4 vs. 10.9 ± 0.2 mmol/liter cell water) and intracellular potassium concentration higher (149.8 ± 1.5 vs. 137.2 ± 1.2 mmol/liter cell water) in erythrocytes from steroid-treated patients. In spite of a significantly decreased intracellular sodium which normally diminishes ouabain-sensitive ^{42}K influx, the ouabain-sensitive K influx was unchanged in erythrocytes from the steroid-treated group. Maximum [^3H]ouabain binding was markedly higher in the steroid-treated group (835 ± 44 vs. 449 ± 11 sites/cell). There was close linear correlation between [^3H]ouabain binding and inhibition of K pump, suggesting the specificity of ouabain binding to Na-K pump sites on the cell membrane. Association kinetics for ouabain were similar in the two groups despite the marked difference in the amount of [^3H]ouabain binding. External potassium concentration required for half-maximum ouabain-sensitive K influx was identical in the two groups. Thus, the additional Na-K pump sites in the steroid-treated group were qualitatively similar to those in normals. These results suggest that administration of glucocorticoids leads to an increase in the number of Na-K pump sites. The increase in the number of Na-K pump sites may explain the low levels of intracellular sodium and higher cell potassium observed in steroid-treated subjects.

A preliminary report of this work was presented at the Annual Meeting of the American Federation of Clinical Research, May 1980, and appeared in abstract form in 1980. (*Clin. Res.* 28: 269.)

INTRODUCTION

A large body of evidence supports the concept that the intracellular concentration of sodium and potassium and the transcellular movement of these monovalent cations are influenced by a specific transport system, consisting of an ATPase and usually referred to as the sodium potassium pump (1-3). Glucocorticoids have been shown to increase Na-K ATPase activity in the rat kidney (4, 5) and more recently in the small and large intestine (6). Erythrocytes from two patients with Cushing's syndrome have also been reported to show increased Na-K ATPase activity (7) but the effects of glucocorticoids on the Na-K pump in human subjects have not been extensively investigated.

The present studies were designed to evaluate the influence of glucocorticoids on the Na-K pump of erythrocytes from human subjects. Previous investigators have used [^3H]ouabain to quantitate the number of Na-K pump sites on various cell types in the healthy state (8-10). The use of this radioligand permitted us to quantitate the number of Na-K pump sites on human erythrocytes and to correlate changes in the number of Na-K pump sites with observed alterations in intracellular electrolytes and potassium flux across cell membranes. The human erythrocyte was chosen because of its known contribution to the elucidation of the normal physiology of the Na-K pump and its easy accessibility in human subjects. Our results indicate a consistent and marked alteration in the Na-K pump of human erythrocytes following the administration of glucocorticoid hormones.

METHODS

Subjects

Subjects were divided into two categories.

Group I. Normal group. This group consisted of 24 healthy adults, 20 males and 4 females, none of whom was on any

drugs. Their ages ranged from 29 to 44 yr (mean age, 36 yr).

Group II. Steroid-treated group. This group consisted of 17 patients, 15 males and 2 females. Their ages ranged from 28 to 82 yr (mean age, 48 yr). Subjects in the steroid-treated group were selected for inclusion in the study if they were receiving 20 mg/d prednisone (or other glucocorticoids with its equivalent dose) for 4 wk or more and if cardiac glycosides were not administered in the week before the study. Blood transfusion within 8 wk before the study was an indication for exclusion. Six subjects were studied before and during glucocorticoid administration.

Solution and chemicals

Incubation medium for [³H]ouabain binding consisted of a K-free solution containing 130 mM NaCl, 10 mM glucose, 10 mM Tris·HCl, 20 mM sucrose, pH 7.45, at 37°C (Na-T-G)¹ with 0.1 μM [³H]ouabain. The effective osmolarity of the solution, measured without glucose and Tris, was 282 ± 10 mosmol/kg water (Fiske osmometer, Fiske Associates, Inc., Uxbridge, Mass.). Standard incubation medium for ⁴²K influx consisted of Na-T-G solution with KCl 10 mM substituted in equimolar amounts for NaCl.

[³H]Ouabain was obtained as dry powder (New England Nuclear, Boston, Mass.) and dissolved in 10 mM Tris·HCl buffer to obtain an aqueous solution. The concentration of ouabain in the sample was measured by Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at 220 nm and the specific activity was determined initially by assuming that all the ³H activity resided in ouabain. This assumption was checked by measuring the reduction in radioactivity bound to erythrocytes when various amounts of unlabeled ouabain were added to the Na-T-G solution with 0.1 μM [³H]ouabain. The specific activity varied from 15.9 to 15.2 Ci/mmol. ⁴²K was obtained as chloride from New England Nuclear. Unlabeled ouabain was obtained from Sigma Chemical Co., St. Louis, Mo. All other solutions were prepared from standard reagent grade chemicals.

Preparation of erythrocyte suspensions

Fresh heparinized blood was centrifuged within 1 h of collection. The plasma, buffy coat, and the topmost layer (= 10%) of the erythrocytes were discarded, and the remaining erythrocytes were resuspended in ice-cold isotonic magnesium chloride, pH 7.4. During each of the three washes that followed, the supernatant MgCl₂ and the topmost layer of erythrocytes were discarded. The precise hematocrit of the washed erythrocyte suspension was determined in triplicate.

Measurement of erythrocyte cation concentrations

The method used was that of Smith and Samuels (11). The erythrocyte suspension of known hematocrit and volume (usually 0.5 ml) was lysed, diluted with lithium diluent, and the cation contents determined with a flame photometer (Instrumentation Laboratory Inc., Lexington, Mass.) and calculated as millimoles per liter of erythrocytes. The volume

fraction of water was calculated from wet and dry weights of cell suspension and specific gravity using the relation published previously (12). Cation concentrations were calculated and expressed as millimoles per liter cell water.

Measurement of K influx

The method used was that of Sachs and Welt (13). Cells were suspended at 2–4% hematocrit in Na-T-G solution containing ⁴²K and 10 mM KCl for 45 min. The reaction was stopped by adding 10 ml of ice-cold isotonic MgCl₂ to the cell suspension. The cells were washed three times at 4°C by alternate centrifugation and resuspension in isotonic MgCl₂. Radioactivities of cell suspensions and incubation media were determined with a gamma counter (Beckman Instruments, Inc.). Similar experiments were carried out in the presence of 0.5 mM ouabain to measure ouabain-insensitive K influx. Ouabain-sensitive ⁴²K influx was calculated as the difference between total and ouabain-insensitive flux. Ouabain-sensitive K influx was measured at various external K concentrations from 0.25 to 10 mmol/liter and the concentration of external potassium required for half-maximal pump influx was determined.

Measurement of [³H]ouabain binding

The method used was that of Joiner and Lauf (14). Pre-warmed [³H]ouabain was added to a 0.2-ml cell suspension in 2 ml of K-free Na-T-G solution at 37°C to give a final concentration of 0.1 μM [³H]ouabain. Studies were performed at least in duplicate and in addition, one study was always performed in the presence of unlabeled ouabain at a concentration of 0.1 mM or more. Maximum ouabain binding was measured after 3 h of incubation, at which time 0.5-ml aliquots were transferred to a tube containing 9 ml of isotonic ice-cold MgCl₂, pH 7.45, over 2 ml of dibutylphthalate. Upon centrifugation, cells with bound radioactivity settled beneath the organic layer, whereas unbound ouabain remained in the aqueous phase. The aqueous layer was aspirated and traces of [³H]ouabain remaining at the side of the tube were removed by carefully pouring fresh MgCl₂ up to the brim and aspirating the MgCl₂ layer. After aspirating both the organic and aqueous layers, the cells were hemolyzed in ice-cold 10 mM Tris·HCl, pH 7.45, and washed twice by centrifugation and resuspension. The cell membranes were solubilized in 0.1 N NaOH, and the β-emission counted in a Beckman liquid scintillation counter using Aquasol (New England Nuclear). [³H]Ouabain binding sites per cell were calculated by using relations published previously (14). Counts obtained in the presence of 0.5 mM unlabeled ouabain were subtracted for this calculation and never amounted to >2% of the total counts.

Kinetics of ouabain binding

[³H]Ouabain binding studies were carried out as described above, and aliquots of cells were withdrawn at various times for determination of [³H]ouabain binding. Maximum [³H]ouabain binding was always measured on these cells by incubation with 0.1 μM ouabain for 3 h.

Correlation between [³H]ouabain binding and ⁴²K influx

To examine the correlation between [³H]ouabain binding

¹ Abbreviation used in this paper: Na-T-G solution, 130 mM NaCl, 10 mM glucose, 10 mM Tris·HCl, and 20 mM sucrose, pH 7.45.

and ^{42}K influx, ^{42}K influx was first measured on cells incubated without [^3H]ouabain. Cells were then suspended in K-free Na-T-G solution with [^3H]ouabain as described above. At appropriate times, before maximum ouabain binding occurred, aliquots were withdrawn in quadruplicate. Duplicate samples were layered over MgCl_2 and dibutylphthalate and the amount of [^3H]ouabain bound was determined as above. The other two samples were washed free of supernatant unbound [^3H]ouabain with ice-cold isotonic MgCl_2 (three washes) and suspended in Na-T-G solution with 10 mM KCl to measure residual ^{42}K influx remaining after partial occupation of Na-K pump sites with ouabain. A few experiments were carried out using 10 nM [^3H]ouabain. In these experiments, maximum ouabain binding was measured at 3 h in solutions containing 0.1 μM ouabain.

Erythrocyte glutamate oxaloacetate transaminase was measured by the method of Beutler (15). Erythrocyte glucose-6-phosphate dehydrogenase was measured by the change in optical density at 340 nM due to NADP reduction using the Sigma test kit. Reticulocytes were stained with a methylene blue stain (Sigma Chemical Co.) and counted with at least 1,000 erythrocytes.

RESULTS

The clinical data for the steroid-treated group are summarized in Table I.

Intracellular cation concentrations (Fig. 1). Intracellular sodium and potassium concentrations in the normal group were 10.9 ± 0.23 and 137.2 ± 1.2 mmol/liter cell water, respectively (Fig. 1). Intracellular sodium was 7.2 ± 0.4 mmol/liter cell water in the steroid-treated group, significantly decreased as compared with normals ($P < 0.001$). Intracellular potassium was 149.8 ± 1.5 mmol/liter cell water, significantly

increased as compared with the normal group ($P < 0.001$). Water content was identical (0.7 liter/liter cells) in both groups.

K influx (Table II). K influx was measured in 10 subjects from the steroid-treated group and 10 subjects from the normal group. The mean intracellular sodium concentrations in these 10 subjects from groups I and II were 11.1 ± 0.56 and 7.4 ± 0.46 mmol/liter cell water, respectively, similar to the averages for the entire group. Several groups of investigators have shown that an increase in intracellular sodium concentration increases, and a decrease in intracellular sodium concentration decreases ouabain-sensitive K influx (K pump influx) (16–19). However, in spite of a significantly lower intracellular sodium concentration in the steroid-treated group, K pump influx was the same as that found in the normal group (Table II). The external potassium concentration required for half-maximal K pump influx was 1.1 mmol/liter in both groups. Ouabain-insensitive K influx was significantly increased in the steroid-treated group as compared with the normal group (0.84 mmol/liter cells per h vs. 0.56 mmol/liter cells per h, $P < 0.001$) (Table II).

Binding of [^3H]ouabain to intact human erythrocytes. At a ligand concentration of 0.1 μM , the specific binding of [^3H]ouabain by erythrocytes from patients receiving glucocorticoids was 15.44 pmol/ml cells (835 ± 44 sites/cells), significantly higher than the 8.57 pmol/ml cells (449 ± 11 sites/cell) by erythrocytes from normal subjects (Fig. 2).

Ouabain binding was rapid at 0.1 μM [^3H]ouabain,

TABLE I
Clinical Characteristics of Patients from the Steroid-treated Group

Patient	Age	Sex	Clinical impression	Medications
1	56	M	Membranous nephropathy	Prednisone 40 mg/d, methyl dopa, furosemide
2	65	M	Temporal arteritis	Prednisone 30 mg/d, cimetidine
3	28	M	Acute leukemia	Prednisone 100 mg/d, isoniazid, ethambutol, nystatin (local)
4	29	M	Sarcoidosis, hypertension, chronic renal failure	Prednisone 60 mg/d, propranolol, hydralazine, prazosin
5	32	F	Midline granuloma	Prednisone 60 mg/d, radiation
6	62	M	Carcinoma of prostate with metastasis	Dexamethasone 8 mg/d, diethylstilbestrol
7	56	F	Carcinoma of breast	Prednisone 30 mg/d, tamoxifen
8	40	M	Accelerated hypertension, intracranial bleeding	Dexamethasone 8 mg/d, propranolol, hydralazine
9	44	M	Membranoproliferative glomerulonephritis	Prednisone 60 mg/d, propranolol
10	43	M	Goodpasture's syndrome	Prednisone 60 mg/d, methyl dopa, hydralazine, furosemide
11	52	M	Renal transplantation	Prednisone 20 mg/d, azathioprine, isoniazid, prazosin
12	82	M	Carcinoma of prostate	Prednisone 60 mg/d, methyl dopa, furosemide
13	43	M	Hydralazine-induced lupus syndrome	Prednisone 60 mg/d, methyl dopa, furosemide
14	28	M	Renal transplantation	Prednisone 40 mg/d, azathioprine
15	59	M	Rheumatoid arthritis	Prednisone 30 mg/d
16	56	M	Extracapillary glomerulonephritis	Prednisone 60 mg/d
17	42	M	Membranous nephropathy	Prednisone 60 mg/d, calcium carbonate

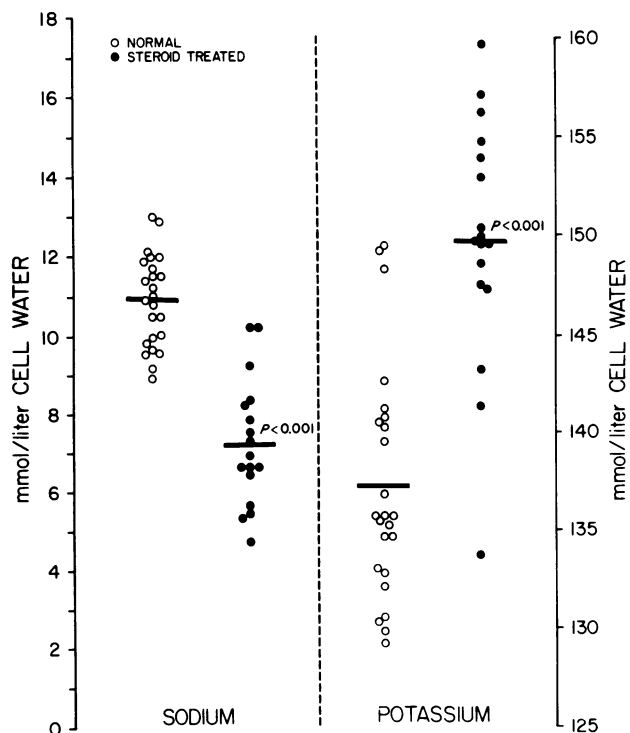
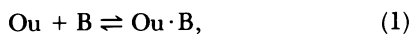


FIGURE 1 Intracellular sodium and potassium concentration in erythrocytes from a group of 24 normal subjects and 17 steroid-treated subjects.

half-maximal binding was established within ~10 min in both groups and binding was essentially complete at 60 min in each and every subject in either group (Fig. 3, top panel).

The interaction of ouabain with its receptor on the Na-K pump could be described by the second order reaction.



where Ou stands for free ouabain, B stands for free ouabain binding sites, Ou·B stands for bound ouabain. Ou·B is relatively stable, since its dissociation rate is very slow (10). Thus, the velocity of ouabain binding was given by the relation,

$$\frac{dx}{dt} = k_b(a - x)(b - x), \quad (2)$$

where a = initial concentration of free ouabain, b = initial concentration of free ouabain binding sites, x = concentration of bound ouabain, and t = time in minutes. The association rate constant k_b was given by integrating and rearranging (2).

$$k_b = \frac{1}{(a - b)t} \ln \frac{b(a - x)}{a(b - x)}. \quad (3)$$

A plot of the value

$$\frac{1}{(a - b)} \ln \frac{b(a - x)}{a(b - x)}$$

plotted against time was linear in both groups. The line for the normal group was $y = -0.01 + 0.07x$, $r^2 = 0.94$ and that for the steroid-treated group was $y = +0.01 + 0.07x$, $r^2 = 0.89$. The two lines were not significantly different. The association rate constant k_b given by the slope of the plots was $0.07 \times 10^7/\text{mol per min}$ in both groups, indicating that the affinity of ouabain for its receptor on the Na-K pump was unchanged in the erythrocytes from the glucocorticoid-treated group in spite of the increase in the total number of Na-K pump sites in the latter group.

Thus, the principal feature of the [^3H]ouabain binding studies was a marked increase in the total number of ouabain binding sites in the erythrocytes from the glucocorticoid-treated group; in other respects the receptor for ouabain was qualitatively identical to the one found in normal subjects.

Correlation of [^3H]ouabain binding and ^{42}K influx. To determine whether the increase in [^3H]ouabain binding in the glucocorticoid-treated group was a reflection of a specific increase in Na-K pump sites or an increase in ouabain binding at nonpump sites, further studies were performed. Aliquots of cells were removed from a bath containing [^3H]ouabain at various times before maximum binding occurred. After washing off supernatant [^3H]ouabain as described earlier, these cells were evaluated for residual K influx. These partially inhibited K pump influxes were measured in order to correlate ouabain binding to K pump inhibi-

TABLE II
Characteristics of K Influx in the Normal and Steroid-treated Group

	Normal	Steroid-treated	P value
Ouabain sensitive			
K influx (mmol/liter cells/h) (External K = 10 mmol/liter)	1.23±0.08 (n = 10)	1.17±0.07 (n = 10)	NS*
Ouabain insensitive			
K influx (mmol/liter cells/h) (External K = 10 mmol/liter)	0.56±0.03 (n = 10)	0.84±0.06 (n = 10)	0.001
$K_{0.5}$ (mmol/liter)	1.1 (n = 5)	1.1 (n = 3)	NS*
Intracellular Sodium (mmol/liter cell water)	11.1 (n = 10)	7.4 (n = 10)	0.001

* Not significant.

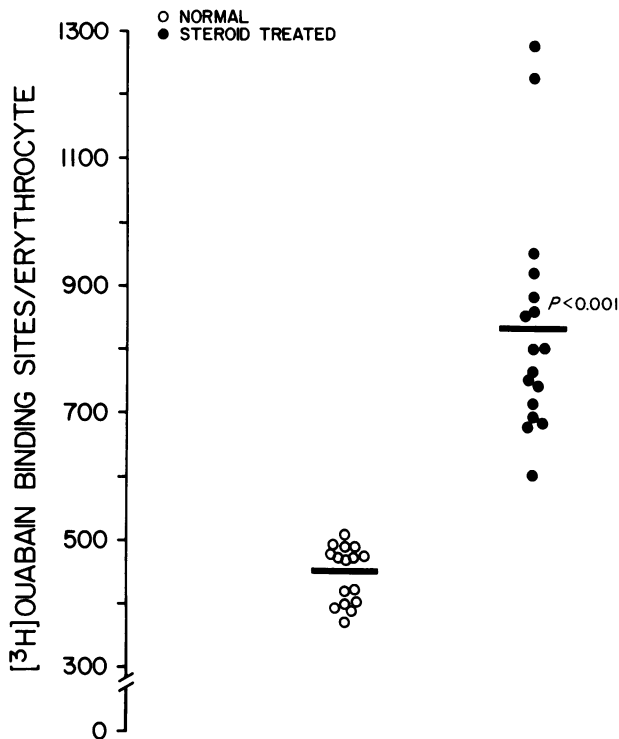


FIGURE 2 $[^3\text{H}]$ ouabain binding sites per cell in a group of 16 normal subjects and 17 subjects from the steroid-treated group. Erythrocytes were incubated in potassium-free Na-T-G solution with $0.1 \mu\text{M}$ $[^3\text{H}]$ ouabain for 3 h. Binding was measured as described in Methods. Counts obtained in the presence of unlabeled ouabain (0.5 mM) were subtracted to obtain specific maximum ouabain binding, which is shown in the figure.

tion as a consequence of the binding of ouabain. ^{42}K pump influx decreased as ouabain binding increased (Figs. 3 and 4). The full complement of receptor site occupancy was required for complete inhibition of K influx (Figs. 3 and 4). No more $[^3\text{H}]$ ouabain binding could be achieved in either group after 100% inhibition of K pump influx, suggesting the absence of non-specific ouabain binding (Fig. 3). The pump influx remaining after partial occupancy of Na-K pump sites by bound $[^3\text{H}]$ ouabain (residual K pump influx, Fig. 3) was subtracted from the total pump influx (uninhibited) present before addition of $[^3\text{H}]$ ouabain at time zero (Fig. 3) to obtain the degree of inhibition of K pump influx. Fig. 4 plots the percent inhibition of K pump influx against the fractional occupancy of $[^3\text{H}]$ ouabain binding sites. The plots were linear in both groups. The linearity of plot in normal subjects confirmed earlier observations of the one-to-one relationship between ouabain molecules bound and Na-K pump sites (10). The linearity of plot in the glucocorticoid-treated group provided evidence for the specificity of $[^3\text{H}]$ ouabain binding in representing additional Na-K pump sites.

Serial measurements after steroid administration. Fig. 5 shows serial measurements of intracellular sodium, potassium, and $[^3\text{H}]$ ouabain binding sites in a patient after administration of a 60 mg/d dose of prednisone. A rise in $[^3\text{H}]$ ouabain binding sites was observed after 4 wk and reached maximum values after 8–12 wk. A fall in the intracellular sodium concentration was observed 2–3 wk after the increase in ouabain binding sites. Both maximum $[^3\text{H}]$ ouabain binding sites and intracellular sodium concentration reached a plateau after 12 wk in spite of continued administration of prednisone in the same dose.

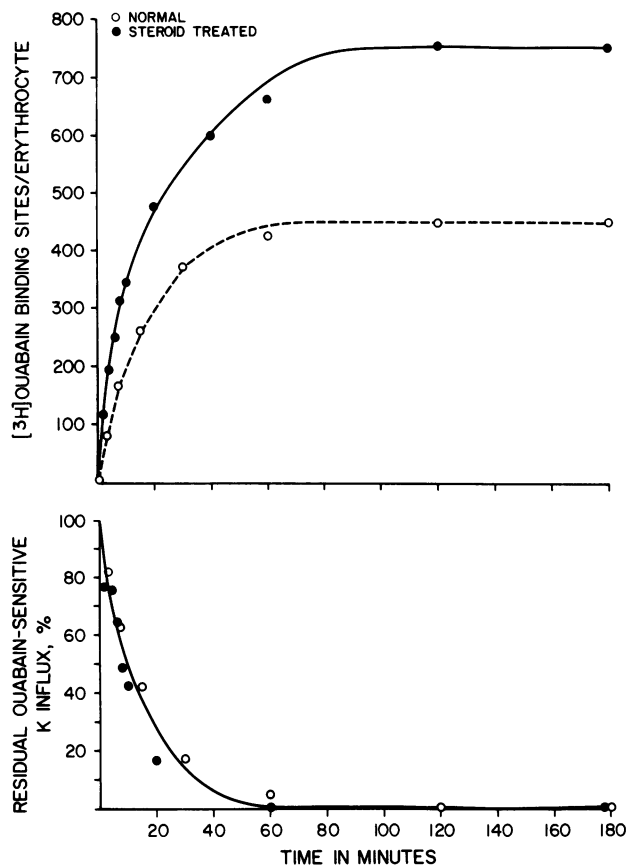


FIGURE 3 Time-course of ouabain binding and ouabain-sensitive potassium influx (K pump influx) in erythrocytes from normal (\circ) and steroid-treated group (\bullet). The experiment was representative of four studies in the normal group and seven studies in the steroid-treated group. Erythrocytes were incubated in a potassium free Na-T-G solution and $[^3\text{H}]$ ouabain at $0.1 \mu\text{M}$ was added at time zero. At the indicated times, samples were taken for ouabain binding (upper panel) and potassium influx (lower panel). Potassium influx was measured in Na-T-G solution containing 10 mmol potassium/liter. The percent residual ouabain-sensitive K influx was derived as

$$\frac{\text{K pump influx, sample}}{\text{K pump influx, uninhibited (time zero)}} \times 100.$$

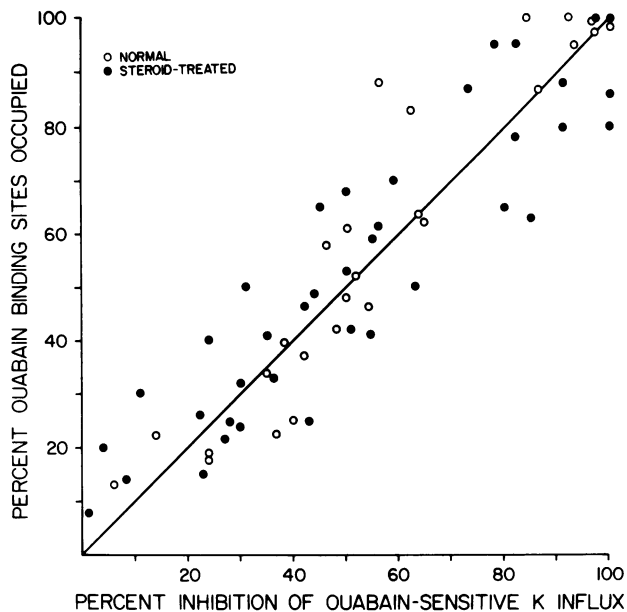


FIGURE 4 Correlation between ouabain binding and inhibition of ouabain-sensitive K influx (K pump influx) in erythrocytes from a group of four normal subjects and seven subjects from the steroid-treated group. Percent inhibition of K pump influx was calculated as:

$$\frac{\text{K pump influx, uninhibited} - \text{K pump influx, sample}}{\text{K pump influx uninhibited}} \times 100$$

(residual K pump influx)

In some experiments, 10 nM ouabain was used to achieve a low percent occupation of ouabain binding sites with accuracy. The line of identity is shown. The line for the normal group was $y = -1.11 + 1.06x$, $r^2 = 0.90$ and that for the steroid-treated group was $y = 4.90 + 0.88x$, $r^2 = 0.88$. These two lines were not statistically different.

Paired studies. In five additional patients, ouabain binding, erythrocyte sodium and potassium concentrations were measured during glucocorticoid treatment and compared with pretreatment values. Ouabain binding increased (845 ± 94 vs. 405 ± 17 sites/cells, $P < 0.01$), erythrocyte sodium decreased (7.4 ± 1.0 vs. 11.4 ± 1.0 mmol/liter cell water, $P < 0.05$), and erythrocyte potassium increased (150.7 ± 3.2 vs. 138.3 ± 1.0 mmol/liter cell water, $P < 0.05$) as compared with pretreatment values. These results suggest that the observed alterations in the Na-K pump in the glucocorticoid group were a result of the administration of glucocorticoids and not due to the disease state.

In vitro incubation of normal erythrocytes with methylprednisolone. Both intact cells and erythrocyte ghosts were incubated in Na-T-G solution containing methylprednisolone sodium succinate 0.1 mM. Intracellular cation concentrations and ^3H binding sites were measured before and up to 24 h after the incubation and compared to control erythrocytes incubated

without the drug. There was no difference in ^3H -ouabain binding sites, net sodium, or potassium flux in the two groups (data not shown).

Morphological and enzymatic characteristics of erythrocytes. To estimate the mean erythrocyte age, we measured several morphological and enzymatic characteristics of the erythrocytes from the two groups. There was no significant difference in the density, mean corpuscular hemoglobin, or the mean corpuscular hemoglobin concentrations in the two groups. Mean corpuscular volume was 89.1 ± 1.2 fl in the steroid-treated group as compared with 87.5 ± 0.2 fl in the normal group ($P < 0.001$). Reticulocyte count was $0.94 \pm 0.16\%$ in whole blood from the steroid-treated group as compared with $0.84 \pm 0.13\%$ in the subjects from normal groups. Erythrocyte glucose-6-phosphate dehydrogenase was 7.67 ± 1.13 U/g hemoglobin in a group of six patients from the steroid-treated group, not significantly different from the normal value of 7.64 ± 1.24 U/g hemoglobin. Erythrocyte glutamate oxaloacetate transaminase level in these patients was 5.29 ± 1.16 U/g hemoglobin as compared with 4.80 ± 1.31 U/g hemoglobin in the normal group (NS). Ouabain binding in these patients was 865 ± 79 sites/cell. There was no correlation between ouabain binding and percent reticulocytes, erythrocyte glucose-6-phosphate dehydrogenase levels or erythrocyte glutamate oxaloacetate transaminase.

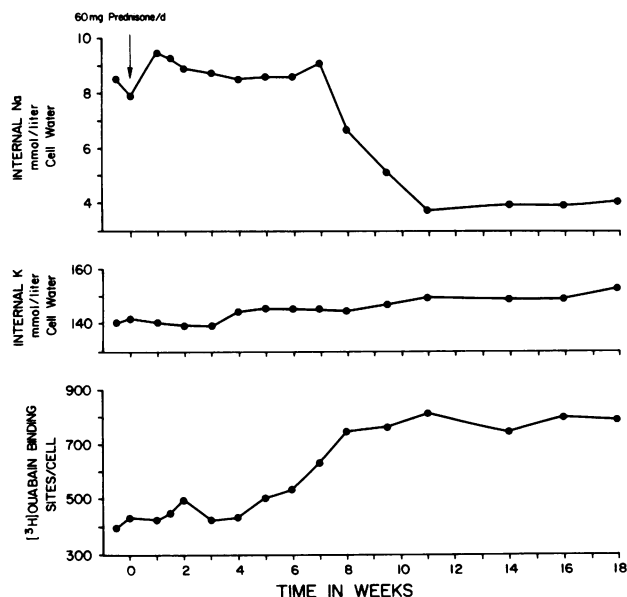


FIGURE 5 Serial measurements of intracellular sodium concentration (upper panel), potassium concentration (middle panel), and ouabain binding sites per cell (lower panel) in a subject receiving 60 mg prednisone/d. Prednisone was started at time zero and continued throughout the 8-wk course in the same dose.

DISCUSSION

Clinical conditions associated with glucocorticoid excess are accompanied by positive sodium balance, hypertension, and hypokalemia. Conversely, deficiency of glucocorticoids, as in Addison's disease or adrenalectomy, is accompanied by sodium loss, hypotension, and hyperkalemia (20). Extensive and elegant investigations have been carried out to define various cellular and subcellular mechanisms of action of glucocorticoid hormones (21). However, relatively little information is available on the effect of glucocorticoids on the Na-K pump in human subjects. In view of the importance of this active pump mechanism in modulating renal and extrarenal transport of potassium and sodium in health and various disease states, we undertook a detailed examination of the effects of exogenous glucocorticoids on the Na-K pump of the human erythrocytes.

An alteration in the activity of the Na-K pump is often reflected by a change in the intracellular concentration of sodium. Thus, in the uremic state and in subjects taking digoxin, a decrease in the activity of the Na-K pump is reflected by an increase in the intracellular sodium concentration (22, 23). Conversely, our initial findings of decreased intracellular sodium concentration and increased intracellular potassium concentration in erythrocytes from the steroid-treated group (Fig. 1) strongly suggest that the activity of the Na-K pump was enhanced. A less attractive possibility is that the reciprocal changes in the intracellular cation concentrations were the result of a simultaneous decrease in the passive permeability of the cell membranes to both sodium and potassium without any significant alteration in Na-K pump activity.

Several groups of investigators have shown that changes in the intracellular concentration of sodium have a direct and almost linear correlation with K pump influx (16–19). Thus, a decrease in intracellular sodium, such as that observed in the steroid-treated group, would be expected to lower K pump influx as compared with normals. However, K pump influx was unchanged in the steroid-treated group despite lower intracellular sodium (Table II), suggesting that K pump influx would be higher in the steroid-treated group when measured under conditions where the internal ionic milieu were comparable in the two groups. The lack of diminution in K pump influx in spite of lower intracellular sodium concentration provides additional evidence of enhanced activity of the Na-K pump in erythrocytes from the steroid-treated group. The finding of an increase in ouabain-insensitive K influx is in keeping with the concept of pump-leak interdependence and is somewhat analogous to other disease states such as hereditary spherocytosis, hereditary stomatocytosis, and in Rh null cells in which similar, concomitant changes in the pump activity and passive fluxes were observed (24–26).

To determine whether increased activity of the Na-K

pump in erythrocytes from the steroid-treated group represented an increased number of Na-K pump sites or increased ion turnover per pump site, [^3H]ouabain binding was measured. The number of ouabain binding sites was significantly higher in the cells from the steroid-treated group. Several lines of evidence suggest that the values for [^3H]ouabain binding reported here represent maximum values for ouabain binding. The concentration of ouabain used was higher than that used by other investigators (27). Concentrations of ouabain $>0.1 \mu\text{M}$ failed to give higher values for [^3H]ouabain binding. At a concentration of $0.1 \mu\text{M}$, ouabain binding reached a plateau and did not change after 1 h (Fig. 3). It was also noteworthy that complete inhibition of K pump influx was observed with the amount of [^3H]ouabain binding reported (Figs. 3 and 4). Increased [^3H]ouabain binding in the glucocorticoid group did not represent an increase in the affinity of ouabain for its receptor on the Na-K pump, since we have shown that the association rate constants for ouabain binding were similar in the glucocorticoid and normal group.

The possibility that increased [^3H]ouabain binding in the glucocorticoid group may be due to a greater proportion of reticulocyte and younger cells in circulation must also be considered, since reticulocyte-rich erythrocyte populations and younger cells separated by density gradients have been shown to have an increase in [^3H]ouabain binding (28, 29). However, the reticulocyte count in our steroid-treated patients was not elevated. Younger cells and reticulocytes have higher activities of erythrocyte glucose-6-phosphate dehydrogenase and glutamate oxaloacetate transaminase (30, 31), whereas the activities of these enzymes were not significantly higher in erythrocytes from steroid-treated patients. There was no correlation between ouabain binding and erythrocyte enzyme levels, indicating that the higher ouabain binding obtained in the steroid-treated group was not due to a younger cell population. Furthermore, younger cells have significantly lower density, higher water content, and lower mean hemoglobin concentration (28, 32–34). Density, water content, and mean hemoglobin concentration were almost identical in the cells from normal and steroid-treated groups. Thus, the observations reported here cannot be attributed to a difference in the age of cells in the two groups, but must be due to other direct effects of glucocorticosteroids on the erythrocytes. Since there was no difference in the morphology of the cells in the two groups and mean corpuscular volume was only slightly higher in the steroid-treated group (89.1 fl as compared with 87.5 fl in the normal group), it is safe to assume that differences in surface area could not account for the increase in [^3H]ouabain binding. Thus, the increased [^3H]ouabain binding in erythrocytes from the steroid-treated group represented an increased density of Na-K pump sites

rather than a major increase in the surface area of the cells.

Several rigorous tests for assessing the specificity of [^3H]ouabain binding to Na-K pump sites of normal erythrocytes have been described by previous workers (8, 10). Using these methods, we attempted to ascertain whether increased [^3H]ouabain binding in the steroid-treated groups represented increased number of Na-K pump sites. There was a linear correlation between the percent occupancy of ouabain binding sites and the percent inhibition of ouabain-sensitive K influx, so that all of the ouabain-sensitive K influx was inhibited when all of the ouabain binding sites were occupied in erythrocytes from both groups (Figs. 3 and 4). No further ouabain binding was observed after complete inhibition of K pump (Fig. 3). The maximum number of binding sites at 3 h was in all cases almost identical to the number obtained from extrapolation of the line correlating binding with inhibition of ouabain-sensitive K influx (Fig. 4). At two different ouabain concentrations (0.1 μM and 10 nM) a given fractional occupancy of ouabain binding sites was associated with the similar fractional inhibition of K pump influx, even though it took considerably longer to occupy the same number of pump sites at lower (10 nM) ouabain concentration (Fig. 4). Thus, the correlation between ouabain binding and the inhibition of K pump influx in both groups was independent of both the time of incubation and the concentration of ouabain used. These findings are similar to those reported earlier by other workers for ouabain binding in normal subjects (10) and strongly suggest that ouabain binding was specific for the Na-K pump sites in erythrocytes from the steroid-treated group as well as normals. In addition, the linearity of the plot (Fig. 4) suggests that all the pump sites in the glucocorticoid group were functionally homogenous with respect to K pump influx.

Using the equation for second order reactions, we calculated the association rate constant (k_b) for the association of ouabain to its binding site in normal subjects and patients from the steroid-treated group. Our finding of similar association rate for the steroid-treated group taken together with the finding of similar $K_{0.5}$ for K influx with respect to external potassium suggests that the ouabain binding and the Na-K pump were qualitatively similar in the two groups and that no conformational change was detectable in the Na-K pump in the steroid group using the methods described.

It is currently believed that glucocorticoids act at the transcriptional or post-transcriptional stage to derepress the genome, allowing RNA translation and protein synthesis to proceed in the nucleus (20). Since the circulating, mature erythrocyte is enucleate and does not synthesize protein and RNA, it is most likely that the observed alterations in the Na-K pump occurred during the course of erythropoiesis and were

primarily directed at the nucleated precursor cells. The observation of a long lag period between the initiation of glucocorticoid administration and the attainment of peak values of [^3H]ouabain binding sites is in agreement with this proposal (Fig. 5). In addition, the alternative but less likely possibility that glucocorticoids act on the mature erythrocyte is not supported by our failure to induce changes in [^3H]ouabain binding or net sodium or potassium flux in erythrocytes and ghosts during *in vitro* incubation in a bath containing methylprednisolone.

Thus, our results indicate that administration of glucocorticoids in pharmacologic doses, given for a wide variety of clinical disorders, consistently results in an increase in the surface density of Na-K pumps on human erythrocytes, an increase in intracellular potassium, and a decrease in intracellular sodium concentration. Our working hypothesis for the observed alterations in Na-K pump invokes an increase in the number of Na-K pump sites as the primary event, with the consequent increase in pump activity resulting in active sodium extrusion up to a point where the lowering of intracellular sodium decreased sodium efflux and potassium influx back to the original steady-state values, albeit in the face of lower intracellular sodium concentration. The finding that increase in [^3H]ouabain binding preceded changes in intracellular electrolytes (Fig. 5) supports this construct. These findings are consistent with earlier observations of an increase in Na-K ATPase in the rat kidney following the administration of glucocorticoids (4) and extend these observations in human subjects. The present data do not allow us to exclude the possibility that these observations may be explained by occupancy of mineralocorticoid receptors by pharmacological doses of glucocorticoids (35). In this context, it is of interest to note that both dexamethasone and aldosterone have been shown to restore Na-K ATPase in adrenalectomized rats but the doses of aldosterone required to restore Na-K ATPase levels in earlier experiments were supermaximal with respect to mineralocorticoid action, leading to the inference that the increase in Na-K ATPase was the result of occupancy of glucocorticoid receptors by aldosterone (4, 36).

Cole and Maletz (37) reported decreased erythrocyte sodium and increased Na-K ATPase in renal transplant recipients, and observed that the changes were the obverse of those reported in uremia. In light of the findings presented here, it appears likely that the findings described in transplant recipients were, in large part, due to the administration of prednisone in those patients. Conventionally, glucocorticoids are thought to have little or no influence on the transport of sodium or potassium in the kidney or the gut. Our finding of consistent and major alterations of the Na-K pump in human erythrocytes during glucocorticoid administration suggests the need to further define a

more precise role of glucocorticoids in modulating electrolyte transport in man.

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