[^{3}H]Ouabain Binding and Na^+,K^+-ATPase in Resealed Human Red Cell Ghosts

D. G. SHOEMAKER and P. K. LAUF

From the Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT The interaction of the cardiac glycoside [^{3}H]ouabain with the Na^+,K^+ pump of resealed human erythrocyte ghosts was investigated. Binding of [^{3}H]ouabain to high intracellular Na^+ ghosts was studied in high extracellular Na^+ media, a condition determined to produce maximal ouabain binding rates. Simultaneous examination of both the number of ouabain molecules bound per ghost and the corresponding inhibition of the Na^+,K^+-ATPase revealed that one molecule of [^{3}H]ouabain inhibited one Na^+,K^+-ATPase complex. Intracellular magnesium or magnesium plus inorganic phosphate produced the lowest ouabain binding rate. Support of ouabain binding by adenosine diphosphate (ADP) was negligible, provided synthesis of adenosine triphosphate (ATP) through the residual adenylate kinase activity was prevented by the adenylate kinase inhibitor Ap_5A. Uridine 5′-triphosphate (UTP) alone did not support ouabain binding after inhibition of the endogenous nucleoside diphosphokinase by trypan blue and depletion of residual ATP by the incorporation of hexokinase and glucose. ATP acting solely at the high-affinity binding site of the Na^+,K^+ pump (K_m ~1 μM) promoted maximal [^{3}H]ouabain binding rates. Failure of 5′-adenylyl-β-γ-imidophosphate (AMP-PNP) to stimulate significantly the rate of ouabain binding suggests that phosphorylation of the pump was required to expose the ouabain receptor.

INTRODUCTION

Previous investigations from this laboratory on human and sheep red blood cells established a one-to-one correlation between the number of molecules of [^{3}H]ouabain bound and the fractional inhibition of the active K^+ influx component of the Na^+,K^+ pump (Joiner and Lauf, 1978*b*). The rate of [^{3}H]ouabain binding was influenced by changes of the intracellular Na^+ and K^+ concentrations, which suggested a conformational dependence of the ouabain receptor similar to or affected by the Na^+,K^+ pump (Joiner and Lauf, 1978*c*). Although others have examined the effect of various ligands on ouabain binding in resealed human erythrocyte ghosts (Lishko et al., 1972; Bodemann and Hoffman, 1976*a–c*), no study has yet determined directly the

Address reprint requests to Dr. Peter K. Lauf, Dept. of Physiology, Duke University Medical Center, Box 3709, Durham, NC 27710.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/83/03/0401/20 $1.00 401
Volume 81 March 1983 401–420

specificity of ouabain for the Na^+,K^+ pump in these model membranes. Moreover, results obtained in resealed ghosts correlating the rate of ouabain binding with the turnover of the Na^+,K^+ pump (Bodemann and Hoffman, 1976*a*) contradict those obtained in intact human and sheep erythrocytes (Joiner and Lauf, 1978*c*), as well as those reported for purified membrane enzyme preparations (Wallick and Schwartz, 1974; Lindenmayer et al., 1974).

We examined the [^{3}H]ouabain binding process in resealed human erythrocyte ghosts under conditions similar to those used by Joiner and Lauf (1978*a*, *b*) in intact cells. We found that binding of one molecule of [^{3}H]-ouabain was sufficient to inhibit one Na^+,K^+-ATPase complex in resealed ghosts. Ouabain binding specifically required ATP and was not supported by other ligands such as Mg^{2+}, inorganic P (P_i), ADP, 5′-adenylyl-β-γ-imidophosphate (AMP-PNP), or uridine 5′-triphosphate (UTP) under the high-sodium conditions used. This work has been reported briefly elsewhere (Shoemaker and Lauf, 1979, 1980).

METHODS

All solutions were prepared with deionized water and reagent grade salts and, when buffered, adjusted to appropriate pH values at 0, 37°C, or room temperature. The solutions used contained 1 mg% chloramphenicol, B grade (Calbiochem-Behring Corp., San Diego, CA), and were: Tris-buffered saline (TBS) 0, 37, and RT: 165 mM NaCl, 2 mM TrisCl, 295 ± 5 mosmol, pH 7.2, at 0, 37, and 22.5°C, respectively; 10 TCl: 10 mM TrisCl, pH 7.6, at 0°C; TCE: 10 mM TrisCl, 0.0625 mM EGTA, pH 7.6, at 0°C; HS-IA (red ghost hemolysing solution): 2 mM ATP, 4 mM $MgSO_4$, pH 3.6–4.0; HS-IB (pink ghost and white ghost primary hemolysing solution): 4 mM $MgCl_2$, 0.1 mM EGTA, pH 3.6–4.0; HS-II (white ghost secondary hemolysing solution): 4 mM $MgCl_2$, 0.1 mM EGTA, pH 6.0; CHB (column hemolysis buffer): 5 mM $MgCl_2$, 15 mM HEPES, 0.1 mM EGTA, pH 6.0; HBS 0, 37, and RT: 150 mM NaCl, 10 mM HEPES, pH 7.4, at 0, 37, and 22.5°C, respectively. Restoring solutions: a 2-M NaCl solution together with various amounts of Tris-OH was used to bring the ghost preparations to isotonicity and pH 7.2.

Inhibitor, Nucleoside Phosphate, and Enzyme Solutions

Ouabain octahydrate, 10^{-3} M, (Sigma Chemical Co., St. Louis, MO) was dissolved in TBS 37 or in deionized water. Two 5-mCi batches of [^{3}H]ouabain (New England Nuclear, Boston, MA) had specific activities of 20.6 and 22.1 Ci/mmol, determined by the method of Joiner and Lauf (1978*a*). 1-mCi/ml stock solutions of [^{3}H]ouabain were prepared by evaporation of benzene:ethanol and addition of TBS 37. A 10-mM trypan blue (Sigma Chemical Co.) solution, pH 6.0, was prepared and stored at room temperature.

Stock solutions of ADP, grade II ATP, Ap_5A, type VI CTP, and type III UTP (all from Sigma Chemical Co.), and AMP-PNP (ICN Nutritional Biochemicals, Cleveland, OH) were prepared in hemolysing solution, and of vanadium-free ATP (Boehringer Mannheim Biochemicals, Indianapolis, IN) and γ-[^{32}P]ATP (triethylammonium salt; New England Nuclear) in deionized water and stored at −90°C.

Stock solutions of creatine phosphate (CP) and creatine kinase (CK) in hemolysing solution and of hexokinase (all from Sigma Chemical Co.) in CHB were made just before the respective ghost preparations.

Preparation of Erythrocyte Ghosts

Human blood was collected by venipuncture using heparin or Alsevier's solution (Bukantz et al., 1946) as anticoagulants and used immediately or stored at 0–4°C for no longer than 20 h. The methods of ghost preparation were modifications of those originally described by Passow's laboratory (Bodemann and Passow, 1972; Lepke and Passow, 1972; Schwoch and Passow, 1973; Wood and Passow, 1981). Three types of ghosts were defined on the basis of dilution and the appearance of the packed pellet of ghosts due to their residual hemoglobin concentration.

Red Ghosts

Red ghosts were prepared by diluting erythrocytes 27-fold (Funder and Wieth, 1976). A 5-min lysis period in HS-IA was followed by addition of the restoring solution, a 15-min incubation at −0.5°C, and a 1-h resealing period at 37°C. The resealed high Na^+ ghosts were collected after 45 min on a sucrose cushion (43% sucrose; 50 mM NaCl; 25 mM TrisCl; pH 7.2, at 0°C) spun at 27,000 *g*.

Pink Ghosts

Pink ghosts were prepared by an initial 170-fold dilution of red cells in hemolysing solution (HS-IB), and the suspension was left for 5 min in the ghosting chamber at pH 6.0. The ghosts were concentrated for 6 min at 30,000 *g* using a GSA head in a Sorvall RC5-B centrifuge (Dupont Instruments, Newton, CT) with no brake at −2–0°C, resuspended in ~30 ml of supernatant, and returned to the ghosting chamber. Compounds to be incorporated into the ghosts were added, and the suspension (final volume = 33 ml) was stirred for 5 min, followed by the addition of 3 ml of restoring solution and stirring for 15 min. Subsequent steps were identical to the preparation of red resealed ghosts with the exception that the sucrose cushion was eliminated after experiments revealed that all pink ghosts were sucrose impermeable.

White Ghosts

White ghosts were lysed analogously to pink ghosts initially but restored after 5 min to isotonicity and pH 7.2 with 3 ml of restoring solution and equilibrated for 15 min. Ghosts were concentrated and the packed ghosts (in 3-ml volumes) were added directly to HS-II. The volume of this second hemolysis solution ranged from 0.030 to 1.0 liter, depending upon the desired dilution of intracellular constituents. After 5 min at pH 6.0, isotonicity and pH 7.2 were restored.

Column Ghosts

Column ghosts were prepared by the method of Wood (1975; Wood and Passow, 1981) with a modified composition and order of addition of column buffers. A K100/45 column (Pharmacia Fine Chemicals, Piscataway, NJ) was equilibrated with CHB, and subsequently a volume of HBS (equal to 10% of the bed volume) was applied, followed by a 15% suspension of red blood cells washed thrice in HBS (5% of the bed volume). White ghosts were collected directly from the column into an ice-cold container, concentrated, restored to isotonicity, and resealed with the desired internal constituents.

Ghost Parameters

Hemoglobin concentrations were determined at 415 nm in the hemolysate supernatants obtained after the ghost resealing period using an extinction coefficient of 8.132

$(gHb/liter)^{-1}$ at 415 nm. Ghost counting was performed with a model F Coulter Counter (Coulter Electronics Inc., Hialeah, FL). Calculation of mean ghost volumes of red ghosts was based on the number of ghosts per liter, cytocrit, and percent trapped extracellular volume on the same population. Estimates of the mean ghost volume of pink and white resealed ghosts were made from a series of diameter measurements of negatively stained samples prepared for electron microscopic examination as previously described (Ting-Beall et al., 1981).

ATP was measured by the coupled fluorometric assay of Lowry et al. (1964) for concentrations of ATP in the range of 0.1–2.0 mM, and for ATP concentrations below 0.1 mM, a slightly modified version of the luciferin-luciferase assay of Kimmich et al. (1975) was used. Extracts were prepared either by acid elution with 0.51 M perchloric acid and subsequent neutralization with 5.62 M K_2CO_3 or by boiling the sample for 2 min. Intracellular ATP concentrations were calculated from the total ATP in the sample using the mean ghost volume, the ghost fractional water content as determined by the method of Cook (1967), and the number of ghosts per sample.

[^{3}H]Ouabain Binding

At zero time, preequilibrated ghosts were suspended to 25% cytocrit in temperature-equilibrated TBS 37 containing [^{3}H]ouabain with concentrations ranging from 10^{-7} to 10^{-8} M. Lower concentrations of [^{3}H]ouabain gave anomalous binding kinetics which were interpreted as resulting from the deterioration of the ghost preparation over the time required to reach saturation binding (6 h). At various times, triplicate samples of the incubation suspension were squirted into 50 vol of ice-cold TBS 0. During the course of the incubation, duplicate samples were also taken for determination of the number of ghosts per milliliter of incubation suspension. The ghosts were washed five times in TBS 0 to reduce the tritium counts in the membrane pellet to a stable value. Ghosts were then hemolysed by the addition of 10 TCl and washed an additional three to five times to obtain the lowest level of hemoglobin possible. The ghost pellet was digested for 15 min at room temperature in 0.5 ml of 0.1 N NaOH, neutralized with 0.2 ml of 0.5 N HCl and counted in 10 ml Scintillant. Vials were counted for tritium in a model LS-250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA) with an efficiency of 25–30%.

The number of ouabain binding sites per ghost was calculated using the disintegrations per minute (dpm) per sample, the measured specific activity of [^{3}H]ouabain (previous studies determined the stated specific activity of [^{3}H]ouabain [New England Nuclear] to be inaccurate [Joiner and Lauf, 1978*a*]), and the number of ghosts per sample. Nonspecific binding was determined in the presence of a 1,000-fold excess of cold ouabain and never exceeded 2%.

Protein Determination

Membrane protein concentrations were measured by the method of Lowry et al. (1951) with the addition of 1% sodium dodecyl sulfate to all samples and standards.

Na^+,K^+-ATPase Assay

The method of Blostein (1968) was used. The assay media consisted of the following (mM): 10 KCl, 75 NaCl, 0.525 $MgCl_2$, 0.025 EGTA, 10 Tris-Cl, 0.5 ATP, 0.1 ouabain (when present), pH 7.6, in a volume of 0.5 ml. Membranes were added in TCE to give a final concentration of 200 μg/ml in the presence of 0.3 μCi/ml [^{32}P]ATP. Incubations were carried out routinely for 10 min at 37°C.

Binding vs. Inhibition Studies

Two sets of samples were taken at various times, one for determination of the number of [^{3}H]ouabain binding sites, and the other for the fractional activity of the Na^+,K^+-ATPase. Ghosts used for determination of Na^+,K^+-ATPase activity were taken from the [^{3}H]ouabain binding incubation and squirted into ice-cold TCE. The membranes were pelleted at 27,000 *g* for 15 min at 0–4°C and residual hemoglobin was removed by one wash in TBS 0 and three washes in TCE.

Bound ouabain dissociates during the enzyme assay. Hence, the Na^+,K^+-ATPase activity reactivated during the course of the enzyme assay incubation was monitored by the appearance of activity from a ouabain-saturated enzyme. The fractional amount of Na^+,K^+-ATPase reactivated during a 10-min assay was 7.6% and was subtracted from the observed activities to obtain the correct values. The degree of inhibition was calculated from the Na^+,K^+-ATPase activity in membranes exposed to [^{3}H]ouabain and control membranes in the absence of [^{3}H]ouabain.

Substrate Support of [^{3}H]Ouabain Binding

Substrates were added to either the primary or secondary hemolysis media, depending upon acid lability, molecular weight, and expense. The resealing period was shortened to 15 min in experiments using the CP-CK ATP-regenerating system to avoid depletion of the system. [^{3}H]Ouabain incubation times were also varied to obtain binding curves delineating the initial rate of binding.

Two enzymes known to be present in membrane preparations of human red blood cell ghosts are adenylate kinase (Cavieres and Glynn, 1976, 1979) and nucleoside diphosphokinase (Mourad and Parks, 1966; Kaplan and Hollis, 1980). Adenylate kinase catalyzes the reaction ATP + AMP $\rightleftharpoons$ 2ADP, whereas nucleoside diphosphokinase facilitates the $X_1TP + X_2DP \rightleftharpoons X_1DP + X_2TP$ reaction where X_1 and X_2 can be any of a variety of purine or pyrimidine ribo- or deoxyribonucleosides. Ap_5A was shown by Lienhard and Secemski (1973) to be a potent inhibitor of the adenylate kinase from rabbit muscle and also effective in human red blood cell ghosts (Cavieres and Glynn, 1976, 1979; Kennedy et al., 1980). Trypan blue inhibits nucleoside diphosphokinase in resealed human red cell ghosts prepared at very high dilutions of internal constituents (Kaplan and Hollis, 1980).

For the ATP support of ouabain binding, a constant concentration of nucleotides over the incubation time was necessary. The CP-CK ATP-regenerating system first used by Garrahan and Glynn (1967*a*) inside resealed ghosts was chosen. Because of the high molecular weight of CK (82,600; Watts, 1973) and the acid lability of CP (Fiske and Subbarow, 1929), the system was incorporated into the secondary hemolysis media in the preparation of white ghosts. A system comprised of 5 mM CP and 5 U/ml CK was capable of maintaining constant ATP values throughout a 20-min incubation provided the resealing period during the ghosting process was shortened from 60 to 15 min.

RESULTS

Resealed red ghosts were prepared with ~2.0 mM ATP sealed inside initially. Fig. 1 shows that ATP was hydrolysed while the ghosts were prepared; hence, ATP was added in excess to saturate the Na^+,K^+ pump (the condition existing in intact cells) during the ouabain binding step. As only a high-affinity site for ATP ($K_m \cong 1$ μM) is available under the existing experimental conditions

(Moczydlowski and Fortes, 1980, 1981), the 500 μM residual ATP was considered sufficient. This assumption was also supported by the fact that internal ATP higher than 2 mM did not increase further the initial rate of [^{3}H]ouabain binding. Finally, incorporation of 5 mM CP and 5 U/ml CK together with 2 mM ATP did not change the rate of ouabain binding (not shown), which indicates a lack of support by the elevated ADP levels resulting from ATP hydrolysis.

There was a considerable difference in the ghost volume of pink and white resealed ghosts. The mean corpuscular volume of red ghosts was 84.9 ± 5.8

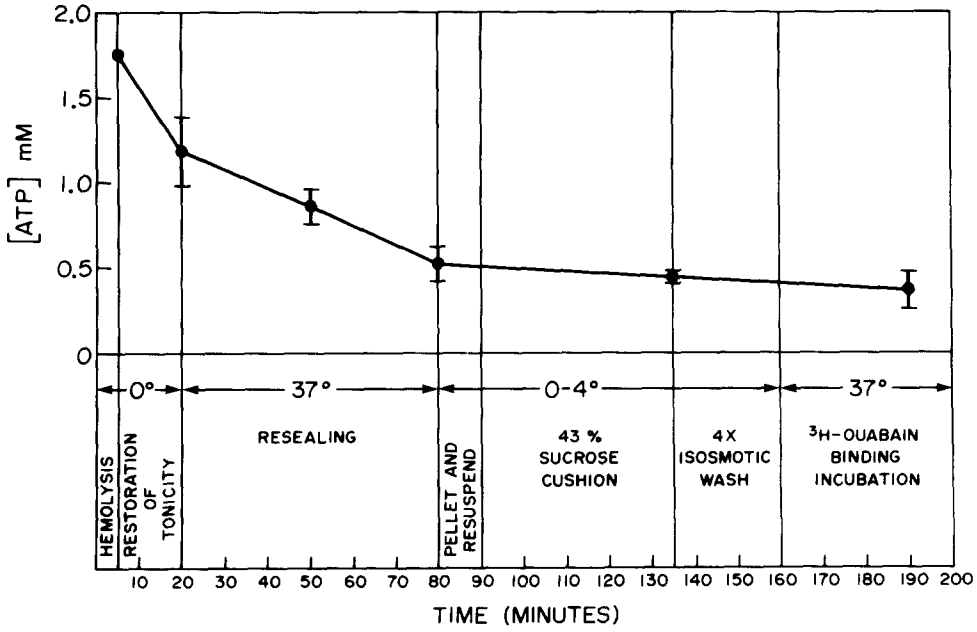

FIGURE 1. Determination of intracellular ATP (millimoles per liter ghost water) throughout the preparation of resealed ghosts and during the [^{3}H]-ouabain binding assay. Red resealed ghosts were prepared to contain 1.75 mM ATP at the time of hemolysis. Points represent the mean ± SEM for triplicate samples. Two other experiments with red ghosts and one with white ghosts gave essentially the same results.

μm^3, whereas pink and white ghosts were smaller and heterogeneous, ranging in volume from 40 to 60 μm^3.

[^{3}H]Ouabain Binding to ATP-replete Ghosts

The rate of [^{3}H]ouabain binding to ATP-replete red, pink, and white resealed ghosts (Fig. 2) revealed identical binding kinetics and the same maximum number of ouabain binding sites per ghost (468 ± 14; n = 5) on a limited sample size (n = 5). Study of a greater sample of individuals over a prolonged time period (3 yr) yielded a range of 400–600 ouabain binding sites/ghost.

The same binding maximum was achieved at each ouabain concentration. Because the nucleotide concentrations within the ghosts could not be controlled for prolonged periods of time, a K_I of the enzyme-ouabain interaction was not obtained. Therefore, the initial rate of ouabain binding was the qualitative tool used to illustrate modulation of the binding process.

[^3H]Ouabain Binding vs. Inhibition of Na^+,K^+-ATPase

We decided to relate ouabain binding to the inhibition of the Na^+,K^+-ATPase activity and not to K^+ pump influx (Dunham and Hoffman, 1971; Joiner and Lauf, 1978*a*) because the passive cation permeabilities in resealed ghosts are known to be approximately four times that of intact red blood cells (Bodemann

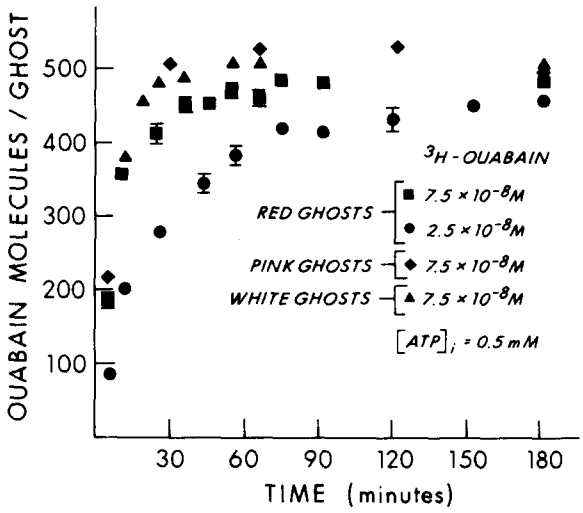

FIGURE 2. The analogous rate of ouabain binding in ATP-replete ([ATP] = 0.4–0.6 mM) red, 200-fold-diluted pink, and 2,000-fold-diluted white resealed ghosts. Also shown is the approach to the same equilibrium level of binding at two different concentrations of [^{3}H]ouabain with the red ghost preparation.

and Hoffman, 1976*a*). Furthermore, the state of the pump substrates once incorporated into resealed ghosts is uncertain. The relationship between the number of ouabain binding sites occupied and the fractional inhibition of the Na^+,K^+-ATPase is shown in Fig. 3: each molecule of bound ouabain inhibited one Na^+,K^+-ATPase complex. The extrapolation to >500 ouabain binding sites/ghost may reflect some dissociation of ouabain during the membrane preparation for Na,K-ATPase assay.

Substrate Support of Ouabain Binding

Before determination of the ATP dependence of ouabain binding, the effect of the reaction products of the Na^+,K^+-ATPase reaction, ADP and P_i, were tested.

Mg and Mg with P_i

Fig. 4 illustrates [^{3}H]ouabain binding to high Na^+ resealed ghosts containing $MgCl_2$ and $MgCl_2$ with P_i. Ghosts containing P_i were assayed in the presence of P_i in the external medium to prevent loss from the ghosts through the anion exchange pathway. It is known that external P_i does not affect the rate of ouabain binding to resealed ghosts (Lishko et al., 1972; Bodemann and Hoffman, 1976*b*). The ouabain binding rate was negligible in comparison with ghosts replete with ATP, but still exceeded the nonspecific binding measured in the presence of 10^3-fold excess of unlabeled ouabain. Results

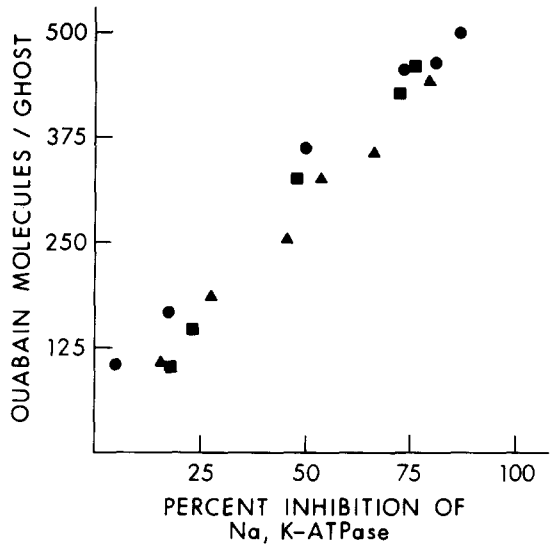

FIGURE 3. Correlation between the fractional occupation of ouabain binding sites with the percent inhibition of Na^+,K^+-ATPase in resealed ghosts membranes derived from red ghosts by hyposmotic lysis. Na^+,K^+-ATPase was assayed using 200 μg/ml membrane protein in a medium which contained (mM): 75 NaCl; 10 KCl; 0.525 $MgCl_2$; 0.025 EGTA; 10 TrisCl; 0.5 ATP; ± 0.1 ouabain; pH 7.6, at 37°C; 0.3 μCi/ml [^{32}P]ATP, sp act = 635 ± 28 nmol P_i/mg protein h. Symbols represent three different experiments under identical conditions.

similar to those shown in Fig. 4 were obtained in two other experiments with pink ghosts. The rate of Mg^{2+}-supported ouabain binding to white ghosts was consistently 50% of that obtained for pink ghosts (not shown), which was due to lower levels of endogenous ATP in white ghosts.

It was found that dilution of intracellular constituents in excess of 20,000-fold in the preparation of white ghosts did not succeed in lowering the ATP concentration within the ghosts to $<3 \times 10^{-7}$ M. Also, column ghosts, which should theoretically contain immeasurable amounts of nucleotide, were routinely found to have ATP concentrations in the range of $3–7 \times 10^{-7}$ M. We propose that this may be a manifestation of the compartmentalized ATP

found by other studies of the human erythrocyte membrane (Proverbio and Hoffman, 1977; Mercer and Dunham, 1981).

ADP

Ouabain binding has previously been shown to be supported by ADP in both human red blood cell membranes (Hoffman, 1969) and purified Na,K-ATPase from calf heart (Schwartz et al., 1968). To exclude the possibility that ADP was supporting ouabain binding by conversion to ATP through the adenylate kinase reaction, the inhibitor Ap_5A was also incorporated into the ghosts along with Mg and ADP. It was first necessary to determine what effect, if any, the compound Ap_5A had on the rate of ouabain binding. Fig. 5 shows the effect of three different concentrations of Ap_5A on the rate of ouabain binding. This

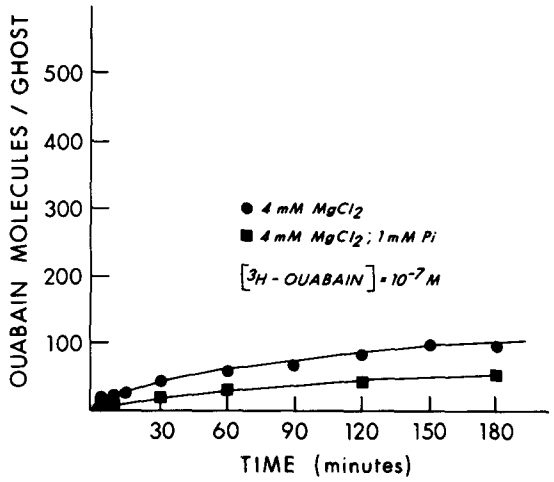

FIGURE 4. The time course of the support of ouabain binding in pink resealed ghosts by Mg^{2+} and Mg^{2+} plus P_i phosphate. Ionic concentrations represent values incorporated into the ghosts at the time of hemolysis. Ghosts containing P_i were assayed in the presence of an equal concentration of extracellular P_i.

significant support of ouabain binding was determined to be due to contaminating ATP in the Ap_5A.

Fig. 6 shows that the rate of ouabain binding to pink ghosts containing both ADP and Ap_5A (upper curve) was caused by the additive effects of ADP (middle curve) and Ap_5A (Fig. 5). In white ghosts with ADP and Ap_5A at a 2,000-fold dilution of original internal constituents, the rate of ouabain binding fell to baseline levels (Fig. 6, bottom curve). The different results obtained in ouabain binding may be due to different dilution of endogenous adenylate kinase in the two ghost preparations. In pink ghosts, the concentration of Ap_5A used was insufficient to inhibit adenylate kinase-supported ATP synthesis from ADP, whereas in the white ghosts, the adenylate kinase was presumably completely inhibited.

To examine whether ATP synthesized via the adenylate kinase was responsible for the ADP support of ouabain binding in pink ghosts, the rate of ouabain binding was followed in white ghosts containing ADP, Ap_5A, and exogenous adenylate kinase (rabbit muscle). Adenylate kinase was present at the levels existing in the pink ghost preparation at a 200-fold dilution of original cell constituents (Cavieres and Glynn, 1979). The rate of ouabain binding obtained in this manner was significantly higher (not shown) than baseline levels.

ATP

The ATP dependence of ouabain binding was examined using the CP-CK-regenerating system to maintain constant ATP levels. To exclude contami-

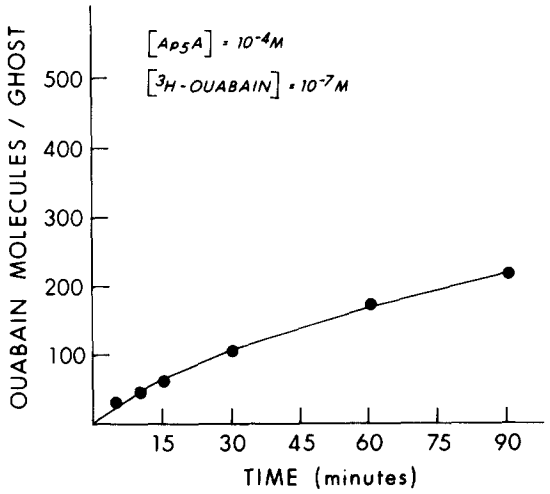

FIGURE 5. The support of ouabain binding to pink resealed ghosts by the incorporation of the adenylate kinase inhibitor Ap_5A inside the ghosts at the hemolysis step. ATP concentrations inside the ghosts loaded with the inhibitor were found to range from 0.3 to 0.5 μM. Experiments performed with 2.0×10^{-4} and 4.0×10^{-4} Ap_5A produced similar binding curves.

nating ATP, the regenerating system itself was examined for its ability to support ouabain binding. As shown in Fig. 7, the CP-CK-regenerating system supported ouabain binding. However, the individual components of the system failed to do so.

To determine whether ouabain binding was supported by the nonhydrolyzable ATP analogue AMP-PNP, the compound was incorporated into resealed ghosts at a concentration sufficient to saturate both the high- and low-affinity ATP sites of the Na,K pump. Although contaminating ATP at a concentration sufficient to promote ouabain binding may obscure the accurate interpretation (Tobin et al., 1974), it is still evident from Fig. 7 that AMP-PNP did not support ouabain binding to the extent shown by ATP at comparable concentrations. Since it is known that AMP-PNP is capable of supporting K:K

exchange by occupation of the low-affinity ATP site, it was then of interest to examine the rate of ouabain binding supported by concentrations of ATP insufficient to act at the low-affinity site.

We explored whether the ATP-regenerating system was incorporated into column ghosts by measuring ouabain binding and ATP levels at indicated intervals, as shown in the lowest binding curve of Fig. 8. Obviously, submicromolar concentrations of ATP supported ouabain binding significantly, which indicates contamination by endogenous ATP. To test this possibility, exogenous ATP was added to the column ghosts just before resealing, and the ATP concentrations were maintained by the regenerating system. Fig. 8 shows that the rate of ouabain binding was significantly affected by changes at submicromolar levels of ATP. Initial experiments revealed that resealed ghosts

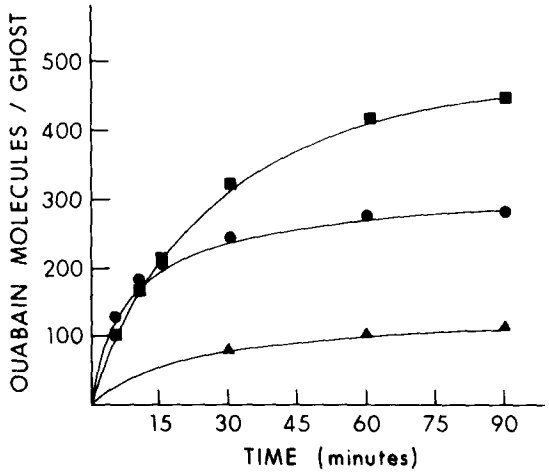

FIGURE 6. The rate of ouabain binding to resealed ghosts under three experimental conditions: ●, pink ghosts loaded with 1.0 mM ADP; ■, pink ghosts loaded with 1.0 mM ADP and 100 μM Ap_5A; ▲, white ghosts loaded with 1.0 mM ADP and 100 μM Ap_5A. The binding curve obtained in the pink ghosts containing only 1.0 mM ADP was representative of two other experiments in pink ghosts and three experiments in white ghosts.

containing between 20 and 200 μM ATP exhibited the same rates of ouabain binding as ghosts containing 500 μM ATP.

UTP and CTP

The nucleotides UTP and CTP have been shown to effectively support ouabain binding to purified Na^+,K^+-ATPase (Tobin et al., 1974) and resealed human erythrocyte ghosts (Hoffman, 1969; Bodemann and Hoffman, 1976*b*). The nucleoside diphosphokinase inhibitor trypan blue was used to investigate whether the support of ouabain binding was the result of synthesis of ATP from the pyrimidine nucleotides through the nucleoside diphosphokinase or of direct interaction of UTP and CTP with the Na^+,K^+ pump.

Initial experiments showed that resealed ghosts containing 2 mM UTP at

the time of resealing supported ouabain binding to an extent almost equivalent to that observed in the presence of ATP. Incorporation of trypan blue did not significantly alter the effect of UTP (Fig. 9), which is consistent with direct support of ouabain binding by UTP or contamination by ATP. To check for ATP contamination, both hexokinase and glucose were included in the resealing mixture, together with UTP and trypan blue. Under these conditions, the rate of ouabain binding was found to be at baseline levels. Ghosts with UTP, glucose, and hexokinase had negligible glucose-6-phosphate content, which excludes UTP as substrate for the hexokinase reaction. Hence,

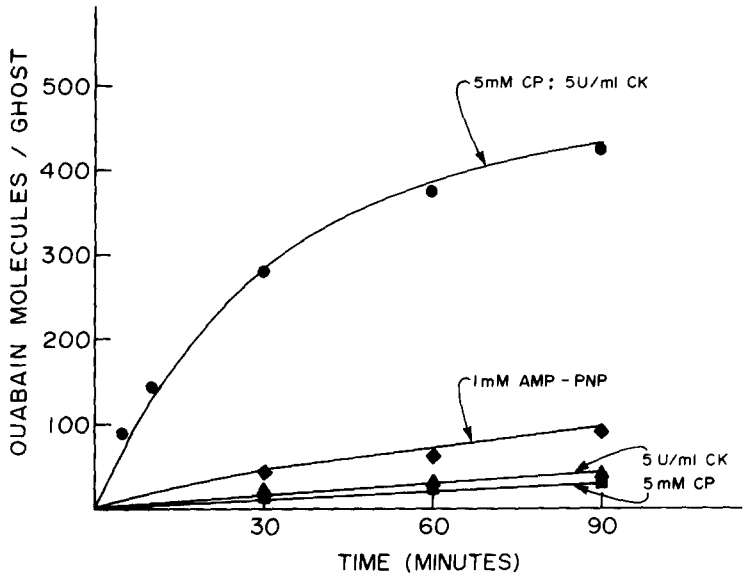

FIGURE 7. Support of ouabain binding in four different preparations of white resealed ghosts loaded with: ■, 5 mM CP; ▲, 5 U/ml CK; ●, 5 mM CP and 5 U/ml CK; and ◆, 1.0 mM AMP-PNP. No exogenous ATP was added to any of these ghost preparations.

UTP did not directly support ouabain binding to the Na,K pump of human red cell ghosts.

This finding was consistent with the relatively low affinity of UTP for purified Na,K-ATPase from guinea pig kidney cortex (Hegyvary and Post, 1971). The pyrimidine nucleotide CTP, however, has a significantly higher affinity for the purified enzyme (Hegyvary and Post, 1971), supports uncoupled Na^+ efflux (Karlish and Glynn, 1974), phosphorylates the enzyme (Tobin et al., 1972), and is hydrolyzed appreciably by the purified enzyme (Robinson, 1982). Fig. 9 shows that the ouabain binding rate in the presence of CTP, trypan blue, glucose, and hexokinase was intermediate to that supported by UTP and ATP.

DISCUSSION

The present study indicates ouabain binding to the Na^+,K^+ pump of resealed ghosts is supported entirely by ATP interaction at its high-affinity site. A linear slope was obtained in a plot of ouabain molecules per ghost vs. inhibition of the Na^+,K^+-ATPase showing that one cardiac glycoside molecule

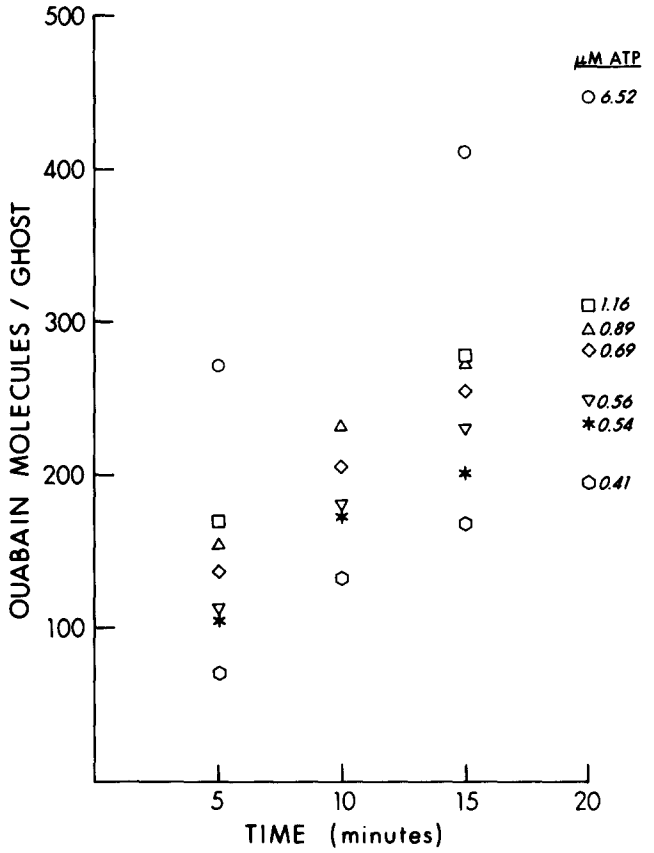

FIGURE 8. The various rates of ouabain binding to column ghosts containing the CP- (5 mM) CK (5 U/ml) ATP-regenerating system. The ATP concentrations (micromoles per liter) are the means of three determinations of 0, 10, and 20 min. The SEM values were 42 for 652, 0.04 for 1.16, and 0.02 for all other ATP values indicated. [^{3}H]Ouabain = 10^{-7} M. Data were obtained on one preparation of column ghosts. Similar results were observed in two other experiments.

is sufficient to inhibit one enzyme complex. Ouabain binding was not supported by (*a*) Mg^{2+}, (*b*) Mg^{2+} plus P_i (under the high-Na^+ conditions), (*c*) Mg^{2+} plus ADP, or (*d*) AMP-PNP, which indicated that phosphorylation of the Na^+,K^+ pump in resealed ghosts was a prerequisite of the enzyme ouabain interaction.

The precipitous decline in the ATP levels of resealed ghosts shown here has been noted previously (Kaplan et al., 1978) and may be due in part to the action of a ouabain-insensitive phosphatase, such as the Mg-ATPase activity of the human erythrocyte membrane (Hoffman, 1980) or to nonspecific phosphatases of the cytosol.

Our verification of the existence of a membrane-associated ATP pool is of relevance in this regard. The previous attempts at quantitation of the amount of ATP associated with this membrane compartment have arrived at values ranging from 100 to 700 molecules of ATP per Na,K pump (Proverbio and Hoffman, 1977; Mercer and Dunham, 1981). Proverbio and Hoffman derived

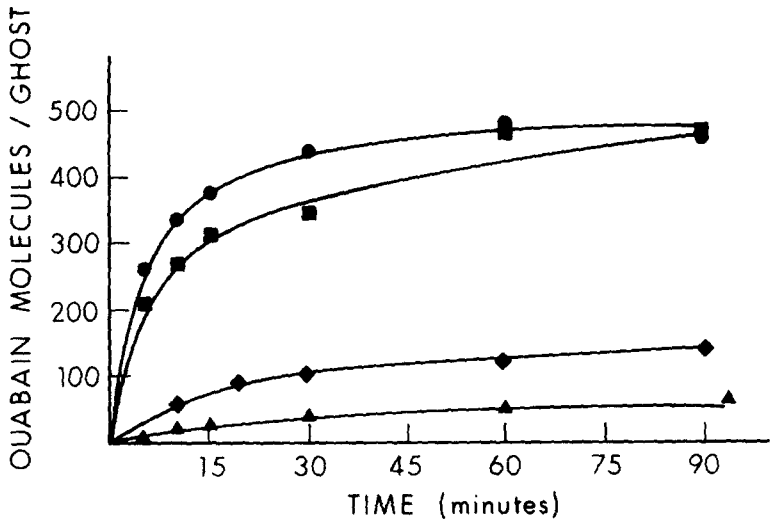

FIGURE 9. The nucleotide support of ouabain binding to three different preparations of column ghosts. Content: ●, 2 mM ATP; 100 μM trypan blue; ■, 2 mM UTP, 100 μM trypan blue; ▲, 2 mM UTP, 100 μM trypan blue, 6 U/ml hexokinase, 10 mM glucose; ◆, 2 mM CTP, 100 μM trypan blue, 6 U/ml hexokinase, 10 mM glucose. The binding curve obtained with 2 mM CTP and 100 μM trypan blue was superimposable upon the 2 mM UTP and 100 μM trypan blue binding curve. [^{3}H]Ouabain = 10^{-7} M.

this estimate from a value of 27,000 molecules of ATP/ghost, which is equivalent to a concentration of 5.0×10^{-7} M ATP/liter ghost water, assuming a value of 85×10^{-15} liter for the mean corpuscular volume. This value corresponds favorably with the ATP associated with the membrane in our preparations of extremely dilute ghosts or column ghosts. In a series of experiments on white ghosts and column ghosts, a range of 0.3–0.7 μM ATP was found to be associated within the human red blood cell ghost when only a fraction of these values should remain as a consequence of the dilution. The mechanism that would explain this finding is one in which ATP is somehow intimately associated with the membrane structure (Mercer and Dunham, 1981).

High Na^+ concentrations are known to inhibit the formation of the Mg^{2+}- and P_i-supported enzyme-ouabain complex (Skou et al., 1971) and to prevent the formation of a Mg^{2+}- and P_i-induced, phosphorylated intermediate of purified Na^+,K^+-ATPase (Post et al., 1975). Despite the rapid decline in ATP and the rise in intracellular ADP and P_i, [^{3}H]ouabain must have been bound to the enzyme supported by Na^+, Mg^{2+}, and ATP. This conclusion was reached because neither Mg^{2+} nor Mg^{2+} plus P_i nor ADP in the presence of sufficient concentrations of the adenylate kinase inhibitor Ap_5A supported significant ouabain binding rates (Figs. 4 and 6).

The integrity of nucleotides in resealed ghosts is crucial for the interpretation of our data. Joiner and Lauf (1978*c*) proposed that a decline in the internal concentration of ATP may have been the cause of the discrepancy in the effect of internal cation concentrations on [^{3}H]ouabain binding in resealed ghosts (Bodemann and Hoffman, 1976*a*) and nystatin-treated red cells (Joiner and Lauf, 1978*c*). The resealed ghost preparation of Bodemann and Hoffman differed from our preparation by significantly lower internal Na^+ concentrations, which may allow for ouabain binding by Na^+, Mg^{2+}, and ATP as well as Mg^{2+} and P_i. Joiner and Lauf (1978*b*, *c*), on the other hand, measured the ATP concentration after the binding was completed to ascertain maintenance of cellular ATP levels.

Ouabain binding in resealed ghosts resembled that of intact human red cells with regard to the apparent irreversibility of the Na^+,K^+ pump-ouabain interaction (Hoffman, 1966, 1969; Dunham and Hoffman, 1971; Sachs, 1974; Joiner and Lauf, 1975, 1978*b*). However, as it was difficult to control internal nucleotide concentrations, the experiments were done with a considerable excess of [^{3}H]ouabain, which precluded measurement of a K_I for the enzyme-ouabain interaction.

Every molecule of [^{3}H]ouabain bound inhibited one Na^+,K^+-ATPase membrane complex, as indicated by the slope of unity in the binding vs. inhibition plot (Fig. 3), which corroborated previous findings in erythrocytes on the correlation between [^{3}H]ouabain binding and K^+ pump flux inhibition (Dunham and Hoffman, 1971; Joiner and Lauf, 1978*a*). Hence, also in ghosts, each Na^+,K^+ pump presents only one ouabain binding site to the external medium.

With the ionic conditions chosen, the Na^+,K^+ pump was either dormant relative to the translocation of cations (Garrahan and Glynn, 1967*a*, *b*; Glynn and Karlish, 1976) or operating in a reduced Na^+/Na^+ exchange mode (Garay and Garrahan, 1973). Nevertheless, the maximum rate of [^{3}H]ouabain binding to these resealed ghost membranes was effected. This was of interest in light of previous reports that the rate of ouabain binding was inversely (Bodemann and Hoffman, 1976*a*) or directly (Joiner and Lauf, 1978*b*) proportional to the rate of turnover of the Na^+,K^+ pump. Hence, the rate of ouabain binding is primarily dependent not on the turnover rate of the Na^+,K^+ pump through a sequence of conformations, but on the existence of a particular reaction intermediate, most probably E_2-P (Sen et al., 1969; Post et al., 1969; Tobin et al., 1973).

Previous evidence for nonspecific support of ouabain binding by various Na^+,K^+-ATPase ligands, substrates, and their analogues has been provided

for purified enzyme preparations (Schwartz et al., 1968; Albers et al., 1968; Tobin et al., 1972) and resealed erythrocyte ghosts (Hoffman, 1969; Bodemann and Hoffman, 1976*b*). Before concluding that the Na^+,K^+ pump-ouabain interaction is related to a particular enzymatic conformation, contaminating activities of adenylate kinase and nucleoside diphosphokinase in resealed ghosts have to be checked. ADP sealed inside high-Na^+ and -Mg^{2+} ghosts supported an initial ouabain binding rate, as did ATP; however, saturation levels were never obtained (Fig. 6). Incorporation of the adenylate kinase inhibitor Ap_5A slightly enhanced the rate of ouabain binding over baseline levels because of hydrolysis of the compound and concomitant ATP production (Fig. 5). However, with ADP plus Ap_5A (at concentrations sufficient to completely inhibit the endogenous adenylate kinase), the rate of ouabain binding was close to that found with Ap_5A alone. Hence, ADP supports ouabain binding via the synthesis of ATP by the adenylate kinase reaction and not by the molecular interaction of ADP with the Na^+,K^+ pump.

Contaminating adenylate kinase activity in preparations of Na^+,K^+-ATPase has been reported previously (Stahl, 1968; Blostein, 1970; Banerjee et al., 1972), and its presence in human red blood cells (Nilsson and Ronquist, 1969; Fajnholc et al., 1972) and resealed ghosts (Cavieres and Glynn, 1976, 1979) is well documented. The observation by Albers et al. (1968) that ouabain binding was supported by ADP can now be explained by the existence of both a contaminating phosphatase and adenylate kinase activities. The studies of Tobin and Sen (1970) as well as Hansen et al. (1971) indicate that ADP inhibits ATP-supported ouabain binding and alone supports the binding to a negligible extent. Therefore, ADP together with Na^+ and Mg^{2+} does not induce the oubain binding conformation of the Na^+,K^+ pump.

Similarly, support of ouabain binding by the pyrimidine triphosphate UTP can also be discounted. UTP sealed inside resealed ghosts at a level previously shown to support ouabain binding in resealed ghosts (Hoffman, 1969; Bodemann and Hoffman, 1976*b*) produced binding curves similar to those obtained with ATP. If the nucleoside diphosphokinase inhibitor trypan blue was introduced together with UTP, however, the rate of ouabain binding was only slightly less than that of UTP-supported binding, which indicates that perhaps UTP does support ouabain binding to the pump. Incorporating 10 mM glucose and hexokinase (to deplete ATP stores), together with UTP, produced baseline level of ouabain binding, which indicates that it was contaminating ATP that supported the rate of ouabain binding initially attributed to the presence of UTP. This finding is consistent with the known low affinity of UTP for the Na,K-ATPase (Hegyvary and Post, 1971; Tobin et al., 1972).

In ATP-depleted column ghosts, CTP produced a slow but significant rate of ouabain binding, a finding consistent with the recent report by Robinson (1982) that demonstrated the Na^+,K^+-CTPase activity to be only 10% of the Na^+,K^+-ATPase activity in a purified enzyme from canine kidney medulla. Tobin et al. (1974), however, found CTP to be as effective as ATP in supporting ouabain binding to a purified Na^+,K^+-ATPase from guinea pig kidney.

Having found that in the presence of high concentrations of Na^+ plus Mg^+ ATP effected ouabain binding, we examined whether ATP was acting at the low- and/or high-affinity sites (Robinson, 1976; Glynn and Karlish, 1976). Initial experiments in which internal ATP concentrations were held constant in the range of 20–200 μM produced maximal rates of ouabain binding. However, when the ATP levels were reduced to submicromolar levels using the column ghosting process, relative rates of ouabain binding became directly proportional to the ATP levels determined. Hence, saturation of the high-affinity ATP binding site in the presence of high Na^+ and Mg^{2+} was sufficient to induce the ouabain binding conformation of the Na^+,K^+ pump, which is consistent with reports on purified Na,K-ATPase from beef brain (Skou et al., 1971).

The results presented emphasize the specific support of ouabain binding by ATP occupying a high-affinity site in resealed ghosts. In the absence of K^+ and in the presence of Na^+, Mg^{2+}, and ATP, the most likely enzymatic intermediate presenting the ouabain binding site to the external media is the E_2-P complex, which is not readily dephosphorylated by ADP.

The authors thank Elizabeth D. Herndon for technical assistance and Evelyne Montaudouin-Daboll and Steffani Webb for typesetting. Supported in part by U. S. Public Health Service grant HL 12,157 from the National Institutes of Health (NIH), and in part by NIH grant AM 28-236.

Received for publication 13 July 1981 and in revised form 20 October 1982.

REFERENCES

Albers, R. W., G. J. Koval, and G. J. Siegel. 1968. Studies on the interaction of ouabain and other cardioactive steroids with sodium-potassium-activated adenosine triphosphatase. *Mol. Pharmacol.* 4:324–336.

Banerjee, S. P., S. M. E. Wong, and A. K. Sen. 1972. Inhibition of sodium- and potassium-dependent adenosine triphosphatase by *N*-ethylmaleimide. II. Effects of sodium-activated transphosphorylation. *Mol. Pharmacol.* 8:18–29.

Blostein, R. 1968. Relationships between erythrocyte membrane phosphorylation and adenosine triphosphate hydrolysis. *J. Biol. Chem.* 243:1957–1965.

Blostein, R. 1970. Sodium-activated adenosine triphosphatase activity of the erythrocyte membrane. *J. Biol. Chem.* 245:270–275.

Bodemann, H. H., and J. F. Hoffman. 1976*a*. Side-dependent effects of internal versus external Na and K on ouabain binding to reconstituted human red blood cell ghosts. *J. Gen. Physiol.* 67:497–525.

Bodemann, H. H., and J. F. Hoffman. 1976*b*. Comparison of the side-dependent effects of Na and K on orthophosphate-, UTP-, and ATP-promoted ouabain binding to reconstituted human red blood cell ghosts. *J. Gen. Physiol.* 67:527–545.

Bodemann, H. H., and J. F. Hoffman. 1976*c*. Effects of Mg and Ca on the side dependencies of Na and K on ouabain binding to red blood cell ghosts and the control of Na transport by internal Mg. *J. Gen. Physiol.* 67:547–561.

Bodemann, H., and H. Passow. 1972. Factors controlling the resealing of the membrane of human erythrocyte ghosts after hypotonic hemolysis. *J. Membr. Biol.* 8:1–26.

Bukantz, S. C., C. R. Rein, and J. F. Kent. 1946. Studies in complement fixation. II. Preservation of sheep's blood in citrate dextrose mixtures (modified Alsevier's solution) for use in the complement fixation reaction. *J. Lab. Clin. Med.* 31:394–399.

Cavieres, J. D., and I. M. Glynn. 1976. Na-Na exchange by the Na pump requires ATP as well as ADP. *J. Physiol. (Lond.).* 263:214–215P.

Cavieres, J. D., and I. M. Glynn. 1979. Sodium-sodium exchange through the sodium pump: the roles of ATP and ADP. *J. Physiol. (Lond.).* 297:637–645.

Cook, J. S. 1967. Nonsolvent water in human erythrocytes. *J. Physiol. (Lond.).* 50:1311–1325.

Dunham, P. B., and J. F. Hoffman. 1971. Active cation transport and ouabain binding in high potassium and low potassium red blood cells of sheep. *J. Gen. Physiol.* 58:94–116.

Fajnholc, N., E. Condrea, and A. De Vries. 1972. Activation of enzymes in red blood cell membranes by a basic protein isolated from cobra venom. *Biochim. Biophys. Acta.* 255:850–857.

Fiske, C. H., and Y. Subbarow. 1929. Phosphocreatine. *J. Biol. Chem.* 81:629–679.

Funder, J., and J. O. Wieth. 1976. Chloride transport in human erythrocytes and ghosts: a quantitative comparison. *J. Physiol. (Lond.).* 262:679–698.

Garay, R. P., and P. J. Garrahan. 1973. The interaction of sodium and potassium with the sodium pump in red cells. *J. Phisiol. (Lond.).* 231:297–325.

Garrahan, P. J., and I. M. Glynn. 1967*a*. Factors affecting the relative magnitudes of the sodium:potassium and sodium:sodium exchanges catalysed by the sodium pump. *J. Physiol. (Lond.).* 192:189–216.

Garrahan, P. J., and I. M. Glynn. 1967*b*. The stoichiometry of the sodium pump. *J. Physiol. (Lond.).* 192:217–235.

Glynn, I. M., and S. J. D. Karlish. 1976. ATP hydrolysis associated with an uncoupled sodium flux through the sodium pump: evidence for allosteric effects of intracellular ATP and extracellular sodium. *J. Physiol. (Lond.).* 256:465–496.

Hansen, O., J. Jensen, and J. G. Nørby. 1971. Mutual exclusion of ATP, ADP and g-strophanthin binding to Na,K-ATPase. *Nat. New Biol.* 234:122–124.

Hegyvary, C., and R. L. Post. 1971. Binding of adenosine triphosphate to sodium and potassium ion-stimulated adenosine triphosphatase. *J. Biol. Chem.* 246:5234–5240.

Hoffman, J. F. 1966. The red cell membrane and the transport of sodium and potassium. *Am. J. Med.* 41:666–680.

Hoffman, J. F. 1969. The interaction between tritiated ouabain and the Na-K pump in red blood cells. *J. Gen. Physiol.* 54:343s–350s.

Hoffman, J. F. 1980. The link between metabolism and active transport of sodium in human red cell ghosts. *J. Membr. Biol.* 57:143–161.

Joiner, C. H., and P. K. Lauf. 1975. The effect of anti-L on ouabain binding to sheep erythrocytes. *J. Membr. Biol.* 21:99–112.

Joiner, C. H., and P. K. Lauf. 1978*a*. Ouabain binding and potassium transport in young and old populations of human red cells. *Membr. Biochem.* 1:187–202.

Joiner, C. H., and P. K. Lauf. 1978*b*. The correlation between ouabain binding and potassium pump inhibition in human and sheep erythrocytes. *J. Physiol. (Lond.).* 283:155–175.

Joiner, C. H., and P. K. Lauf. 1978*c*. Modulation of ouabain binding and potassium pump fluxes by cellular sodium and potassium in human and sheep erythrocytes. *J. Physiol. (Lond.).* 283:177–196.

Kaplan, J. H., B. Forbush III, and J. F. Hoffman. 1978. Rapid photolytic release of adenosine 5′-triphosphate from a protected analogue: utilization by the Na:K pump of human red blood cell ghosts. *Biochemistry.* 17:1929–1935.

Kaplan, J. H., and R. J. Hollis. 1980. External Na dependence of ouabain-sensitive ATP:ADP exchange initiated by photolysis of intracellular caged-ATP in human red cell ghosts. *Nature (Lond.)*. 288:587–589.

Karlish, S. J. D., and I. M. Glynn. 1974. An uncoupled efflux of sodium ions from human red cells, probably associated with Na-dependent ATPase activity. *Ann. NY Acad. Sci.* 242:461–470.

Kennedy, B. G., G. Lunn, and J. F. Hoffman. 1980. Effect of changing ATP/ADP ratios on the operation of the Na:K pump of red blood cell ghosts. *Fed. Proc.* 39:1978.

Kimmich, G. A., J. Randles, and J. S. Brand. 1975. Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. *Anal. Biochem.* 69:187–206.

Lepke, S., and H. Passow. 1972. The effect of pH at hemolysis on the reconstitution of low cation permeability in human erythrocyte ghosts. *Biochim. Biophys. Acta.* 255:696–702.

Lienhard, G. E., and I. I. Secemski. 1973. P^1, P^5-Di(adenosine-5′) pentaphosphate, a potent multisubstrate inhibitor of adenylate kinase. *J. Biol. Chem.* 248:1121–1123.

Lindenmayer, G. E., L. K. Lane, and A. Schwartz. 1974. Effects of sodium and potassium on the partial reactions of a highly purified (Na^+ + K^+)-ATPase: modulation of the rate of ouabain interaction. *Ann. NY Acad. Sci.* 242:235–245.

Lishko, V. K., M. K. Malysheva, and T. I. Grevizirskaya. 1972. The interaction of the (Na^+, K^+)-ATPase of erythrocyte ghosts with ouabain. *Biochim. Biophys. Acta.* 288:103–106.

Lowry, O. H., J. V. Passonneau, F. X. Hasselberger, and D. W. Schulz. 1964. Effect of ischemia on known substrates and cofactors of the glycolytic pathway of brain. *J. Biol. Chem.* 239:18–30.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Mercer, R. W., and P. B. Dunham. 1981. Membrane-bound ATP fuels the Na/K pump. Studies on membrane-bound glycolytic enzymes on inside-out vesicles from human red cell membranes. *J. Gen. Physiol.* 78:547–568.

Moczydlowski, E. G., and P. A. G. Fortes. 1980. Kinetics of cardiac glycoside binding to sodium, potassium adenosine triphosphatase studied with a fluorescent derivative of ouabain. *Biochemistry.* 19:969–977.

Moczydlowski, E. G., and P. A. G. Fortes. 1981. Inhibition of sodium and potassium adenosine triphosphatase by 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene) adenine nucleotides. *J. Biol. Chem.* 256:2357–2366.

Mourad, N., and R. E. Parks. 1966. Erythrocyte nucleoside diphosphokinase. II. Isolation and kinetics. *J. Biol. Chem.* 241:271–278.

Nilsson, O., and G. Ronquist. 1969. Enzyme activities and ultrastructure of a membrane fraction from human erythrocytes. *Biochim. Biophys. Acta.* 183:1–9.

Post, R. L., S. Kume, T. Tobin, B. Orcutt, and A. K. Sen. 1969. Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase. *J. Gen. Physiol.* 54:3065–3265.

Post, R. L., G. Toda, and F. N. Rogers. 1975. Phosphorylation by inorganic phosphate of sodium plus potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* 250:691–701.

Proverbio, F., and J. F. Hoffman. 1977. Membrane compartmentalized ATP and its preferential use by the Na,K-ATPase of human red cell ghosts. *J. Gen. Physiol.* 69:605–632.

Robinson, J. D. 1976. Substrate sites of the (Na^+ + K^+)-dependent ATPase. *Biochim. Biophys. Acta.* 429:1006–1019.

Robinson, J. D. 1982. Differences between CTP and ATP as substrates for the (Na + K)-ATPase. *Arch. Biochem. Biophys.* 213:650–657.

Sachs, J. R. 1974. Interaction of external K, Na, and cardioactive steroids with the Na-K pump of the human red blood cell. *J. Gen. Physiol.* 63:123–143.

Schwartz, A., H. Matsui, and A. H. Laughter. 1968. Tritiated digoxin binding to ($Na^+ + K^+$)-activated adenosine triphosphatase: possible allosteric site. *Science (Wash. DC).* 160:323–325.

Schwoch, G., and H. Passow. 1973. Preparation and properties of human erythrocyte ghosts. *Mol. Cell. Biochem.* 2:197–218.

Sen, A. K., T. Tobin, and R. L. Post. 1969. A cycle for ouabain inhibition of sodium- and potassium-dependent adenosine triphosphatase. *J. Biol. Chem.* 244:6596–6604.

Shoemaker, D. G., and P. K. Lauf. 1979. ^{3}H-ouabain binding and Na,K-ATPase in resealed human red cell ghosts. *Fed. Proc.* 38:1128.

Shoemaker, D. G., and P. K. Lauf. 1980. Effect of ATP, ADP, Pi, and Ap_5A on the rate of ^{3}H-ouabain binding to resealed human red cell ghosts. *Fed. Proc.* 39:2152.

Skou, J. C., K. W. Butler, and O. Hansen. 1971. The effect of magnesium, ATP, P_i, and sodium on the inhibition of the ($Na^+ + K^+$)-activated enzyme system by g-strophanthin. *Biochim. Biophys. Acta.* 241:443–461.

Stahl, W. L. 1968. Sodium stimulated [^{14}C]adenosine diphosphate-adenosine triphosphate exchange activity in brain microsomes. *J. Neurochem.* 15:511–518.

Ting-Beall, H. P., M. J. Costello, D. Shoemaker, and V. F. Holland. 1981. Ultrastructure of hemoglobin-depleted human erythrocyte resealed ghosts. *Biochim. Biophys. Acta.* 640:807–811.

Tobin, T., and A. K. Sen. 1970. Stability and ligand sensitivity of [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase. *Biochem. Biophys. Acta.* 198:120–131.

Tobin, T., T. Akera, S. I. Baskin, and T. M. Brody. 1973. Calcium ion and sodium- and potassium-dependent adenosine triphosphatase: its mechanism of inhibition and identification of the E_1-P intermediate. *Mol. Pharmacol.* 9:336–349.

Tobin, T., T. Akera, C. Y. Lee, and T. M. Brody. 1974. Ouabain binding to ($Na^+ + K^+$)-ATPase. Effects of nucleotide analogues and ethacrynic acid. *Biochim. Biophys. Acta.* 345:102–117.

Tobin, T., S. I. Baskin, T. Akera, and T. M. Brody. 1972. Nucleotide-specificity of the Na^+-stimulated phosphorylation and [^{3}H]ouabain-binding reactions of ($Na^+ + K^+$)-dependent adenosine triphosphatase. *Mol. Pharmacol.* 8:256–263.

Wallick, E. T., and A. Schwartz. 1974. Thermodynamics of the rate on binding of ouabain to the sodium, potassium-adenosine triphosphastase. *J. Biol. Chem.* 249:5141–5147.

Watts, D. C. 1973. Creatine kinase (adenosine 5′-triphosphate-creatine phosphatase. *In* The Enzymes. P. D. Boyer, editor. Academic Press, Inc., New York. 383–455.

Wood, P. G. 1975. Preparation of hemoglobin-free resealable RBC ghosts by a gel filtration technique. *Fed. Proc.* 34:249.

Wood, P. G., and H. Passow. 1981. Techniques for the modification of the interacellular composition of red blood cells. *In* Techniques in Cellular Physiology. P. F. Baker, editor. Elsevier/North-Holland, New York. 1–43.