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Effects of $(Na^+ + K^+)$-ATPase-Specific Antibodies on Enzymatic Activity and Monovalent Cation Transport*

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Summary. Antibodies have been obtained that specifically interact with the transport enzyme $(Na^+ + K^+)$-activated ATPase. The antigen used was purified $(Na^+ + K^+)$-ATPase from canine renal medulla. Purified γ globulin from immunized animals, but not from control animals or preimmune serum, inhibited $(Na^+ + K^+)$-ATPase from canine renal medulla with reduction of activity to 33 ± 4 (SD) % in a concentration-dependent manner. Maximum inhibition occurred in less than 5 minutes at 37 °C. The Mg^{++}-dependent, nonouabain inhibited component of activity (Mg^{++}-ATPase) was unaffected. Fab fragments obtained by papain cleavage of the γ globulin fraction had similar inhibitory activity and specificity. These antibodies also produced varying degrees of concentration-related inhibition of canine myocardial, calf brain, and human red cell ghost $(Na^+ + K^+)$-ATPase, but not Mg^{++}-ATPase activity.

Despite marked inhibition of $(Na^+ + K^+)$-ATPase activity in these enzyme preparations from disrupted cells, experiments with canine renal slices, guinea pig atrial strips, and human red cells showed no specific effect of antibody on ouabain-inhibitable $^{86}Rb^+$ uptake, indicating a lack of inhibition of active monovalent cation transport in the intact cell. When specific antibody had access to the inner surface of lysed, resealed human red cell ghosts, however, complete inhibition of $(Na^+ + K^+)$-ATPase-mediated $^{22}Na^+$ efflux was observed. Consistent with this finding was complete inhibition of $(Na^+ + K^+)$-ATPase activity in inside-out, but not right-side-out, red cell membrane vesicles. These experiments demonstrate immunologic cross-reactivity among $(Na^+ + K^+)$-ATPases from different organs and different species. In addition, they indicate that the antibody response resulting in enzyme and transport inhibition is directed against an antigenic determinant or determinants inaccessible to macromolecules at the outer cell surface.

Substantial interest has focused recently on the localization of cell membrane components in general (Bretscher, 1973*a*; Steck, 1974) and of the monovalent cation transport enzyme complex known as $(Na^+ + K^+)$-ATPase in particular (Marchesi & Palade, 1967; Ruoho & Kyte, 1974). The vectorial nature of Na^+ and K^+ transport as well as the activation of $(Na^+ + K^+)$-ATPase activity by Na^+ only at the

* A preliminary account of parts of this work has been presented previously in abstract form (*J. Clin. Invest.* **52**:78a, 1973).

inner cell surface and by K^+ only at the outer surface (Whittam & Ager, 1964) provide functional evidence of communication of this complex with both inner and outer cell surfaces during the transport cycle. This concept is further strengthened by observations that ATP hydrolysis and inorganic phosphate release occur at the inner cell surface, while cardiac glycoside binding to and inhibition of $(Na^+ + K^+)$-ATPase occur only from the outer cell surface, at least in the case of the human red blood cell (Hoffman, 1966; Perrone & Blostein, 1973) and squid giant axon (Caldwell & Keynes, 1959). Previous studies from our laboratory using macromolecular conjugates of cardiac glycosides (Smith, Wagner, Markis & Young, 1972) and proteolytic enzyme attack (Wagner, Smith & Young, 1974) have suggested that $(Na^+ + K^+)$-ATPase and cardiac glycoside receptors are not readily accessible at the outer cell surface.

Development of methods for the purification of $(Na^+ + K^+)$-ATPase now permits the use of highly purified preparations as antigens to elicit specific antibodies in experimental animals (Jørgensen, Hansen, Glynn & Cavieres, 1973; Kyte, 1974; McCans, Lane, Lindenmayer, Butler & Schwartz, 1974). In the studies to be described, antibodies were obtained from rabbits challenged with $(Na^+ + K^+)$-ATPase from canine renal medulla. The effects of these antibodies on catalytic activity of $(Na^+ + K^+)$-ATPase and on monovalent cation transport have been studied in preparations from various organs and species, with particular attention to the sidedness of the antibody interaction with cell membrane $(Na^+ + K^+)$-ATPase.

Materials and Methods

Preparation of Antigen

$(Na^+ + K^+)$-ATPase was prepared from canine renal medulla essentially according to the method of Kyte (1971, 1972). The supernatant enzyme was passed over a short column of XAD4 resin in order to remove deoxycholate. The purified enzyme showed only two bands of apparent molecular weight 95,000 and 50,000 on SDS-polyacrylamide gel electrophoresis of 30 μg aliquots by the method of Weber and Osborn (1969). The specific activity was 280 μmoles Pi/mg protein/hr, and was fully inhibited by 10^{-4} M ouabain. In view of substantially higher specific activities of $(Na^+ + K^+)$-ATPase from renal medulla obtained by Lane, Copenhaver, Lindenmayer and Schwartz (1973) and by Jørgensen (1974), this suggests some degree of denaturation of the enzyme during preparative procedures, although additional protein contaminants co-electrophoresing on SDS-polyacrylamide gels cannot be excluded with certainty. All protein concentrations were measured by the procedure of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as standard.

Immunologic Methods

$(Na^+ + K^+)$-ATPase was suspended in complete Freund's adjuvant to a final concentration of 0.5 mg enzyme protein per milliliter of emulsion. After preimmune serum samples were obtained, two New Zealand white rabbits (designated A_1 and A_2) were challenged primarily with multiple intradermal injections of approximately 20 μl of antigen into 20 intradermal injection sites in the flanks of each animal. An additional 200 μl were injected into toe pad sites such that each animal received a primary challenge of 300 μg of enzyme protein. In addition to the use of preimmune sera for control experiments, two rabbits (designated A_3 and A_4) of the same strain were challenged in the identical manner, but with 300 μg of protein from human red cell ghosts prepared according to the method of Heinz and Hoffman (1965). All animals were bled at four-weekly intervals by ear artery incision. Twenty weeks after primary challenge, booster injections of 100 μg of enzyme protein in complete Freund's adjuvant were given intramuscularly to each rabbit. These booster injections were continued at four-weekly intervals thereafter.

Blood samples were allowed to clot at room temperature, then were centrifuged for 10 min at $5{,}000 \times g$ to remove formed elements. Serum sodium and potassium concentrations from all bleedings were determined by flame photometry.

Purification of γ globulins from crude antisera was accomplished in all samples by ammonium sulfate precipitation (Deutsch, 1967). Gamma globulin fractions from 12 and 32-week bleedings from rabbit A_1 were additionally subjected to DEAE cellulose chromatography (Deutsch, 1967). Results obtained with antibody globulins which had undergone DEAE cellulose purification were indistinguishable from those after ammonium sulfate precipitation alone, and some subsequent studies were performed with γ globulins isolated by ammonium sulfate precipitation alone. All γ globulin fractions were subjected to dialysis for at least 24 hours against two changes of buffer of identical composition to that to be used in ATPase assays or cation transport experiments, omitting ATP.

Fab fragments of γ globulin fractions were prepared by papain digestion and reaction with iodoacetate as described by Edelman and Marchalonis (1967).

$(Na^+ + K^+)$-ATPase Preparations for Study of Interaction with Antibody

Canine renal medullary $(Na^+ + K^+)$-ATPase was prepared as noted above, except the preparation was carried only through the sodium iodide step in order to provide a particulate enzyme with adequate Mg^{++}-ATPase as well as $(Na^+ + K^+)$-ATPase activity for assessment of specificity of antibody effects. These preparations had ATPase-specific activities in the 150–200 μmoles Pi/mg × hr range, 75–85% of which was inhibited by 10^{-4} M ouabain. With the exception of kinetic studies performed as described using a linked enzyme assay (Albers, Koval & Siegel, 1968), enzymatic activity was determined at 37 °C as previously described (Smith *et al.*, 1972) with measurement of inorganic phosphate cleaved by the method of Fiske and Subbarow (1925). All assays were linear with respect to time and enzyme concentration over the ranges studied.

Canine myocardial microsomal $(Na^+ + K^+)$-ATPase was prepared by minor modification of the method of Schwartz *et al.* (1971). Solubilization of this preparation with 0.2% Lubrol-WX has been described in detail elsewhere (Smith, Wagner, Strosberg & Young, 1974*a*; Smith, Wagner & Young, 1974*b*). Briefly, microsomal enzyme prepared by deoxycholate treatment of myocardial homogenates was suspended in 0.32 M sucrose, 1.0 mM H_4EDTA, pH 7.0, with Tris base at a concentration of 2 mg protein/ml. An equal volume of 0.4% (w/v) Lubrol WX in the same buffer was then added. The mixture was homogenized with 10 strokes in a ground-glass homogenizer and allowed to stand at 0 °C. At the end of 30 min, the mixture was centrifuged at $100{,}000 \times g$ for 1 hour. The supernatant

solution was carefully removed and used as "solubilized" enzyme. The specific activity of the solubilized enzyme was 12.3 μmoles Pi/mg × hr and 82% of the total ATPase activity was inhibited by ouabain. Corresponding values for the microsomes prior to solubilization were 8.0 μmoles Pi/mg × hr, and 41% inhibition by 10^{-4} M ouabain.

Calf brain microsomal $(Na^+ + K^+)$-ATPase was prepared according to the method of Uesugi *et al.* (1969). The specific activity of the preparation used in the present studies was 14.6 μmoles Pi/mg × hr. All activities were determined by colorimetric measurement of inorganic phosphate cleaved, as noted above.

Fresh human red blood cells obtained by venipuncture were subjected to osmotic lysis and washing according to the method of Dodge, Mitchell and Hanahan (1963). To ensure full substrate access, three cycles of freezing and thawing were carried out before measurement of $(Na^+ + K^+)$-ATPase activity using γ-^{32}P ATP (12–15 Ci/mmole, New England Nuclear Corp., Boston, Mass.) as described previously (Smith *et al.*, 1972).

$^{86}Rb^+$ *Transport in Intact Cells*

Three types of preparations were used to study the effects of antibodies on monovalent cation active transport.

Canine Renal Medullary Slices. Kidneys obtained immediately after sacrifice of pentobarbital-anesthetized dogs were cooled in iced saline slush. After dissection at 4 °C to expose the outer red medulla, slices were made with a Stadie-Riggs microtome and were placed in physiologic buffer containing, in mM concentrations: KCl, 4.0; NaCl, 120; $NaHCO_3$, 24; $MgCl_2$, 2.0; $CaCl_2$, 2.5; glucose, 5.6; and Na phosphate buffer, 1.1 mM, adjusted to pH 7.4 at 30 °C.

After a 15-min period of equilibration in this medium at 30°, slices were transferred to beakers containing the same buffer medium, except that the KCl concentration was adjusted to 2 mM and ^{86}RbCl was added to give count rates of about 10^6 DPM per milliliter of medium. Carrier RbCl was also present to give a final Rb^+ concentration of 0.1 mM. Uptake of $^{86}Rb^+$ was determined under four sets of conditions: (1) as described, with no added components, (2) as described, with the addition of 10^{-4} M ouabain to the medium, (3) as described, with the addition of 2 mg per ml of specific γ globulin from the 32 week bleedings of rabbits A_1 or A_2, and (4) as described, with the addition of 2 mg per ml of control γ globulin from rabbit A_3 or from a preimmune bleeding from rabbit A_2.

Duplicate slices were removed from the incubation medium immediately after immersion and at 15, 30, 45, and 60 min. These slices were rinsed in four successive beakers containing 200 ml physiologic buffer at 30 °C for 5 sec each. They were then counted individually in 1 ml of physiologic buffer in a Nuclear-Chicago gamma well counter and immediately thereafter blotted and weighed. Determinations under each set of conditions were repeated twice.

Guinea Pig Left Atrial Strips. Adult guinea pigs of both sexes were killed by cervical dislocation and the beating hearts removed. Left atria were dissected free and divided into two strips of equal size which were placed in physiologic buffer identical to that described for experiments with canine renal medullary slices. A fine thread was attached to a corner of each atrial strip to facilitate transfers between vessels. After equilibration at 30 °C for 15 min, atrial strips were exposed to media exactly as described for the canine renal medulla slice experiments. Immediately after immersion and at 15, 30, 45, and 60 min each strip was rinsed in four successive beakers containing 200 ml physiologic buffer and counted in a gamma well counter for 1 min in 1 ml of buffer warmed to 30 °C. Immediately after counting, slices were returned to the incubation buffer containing $^{86}Rb^+$ and incubated with gentle stirring until the next counting time. After the final counting at 60 min, each atrial strip was blotted and weighed.

Human Red Blood Cells. Fresh human red blood cells obtained from normal volunteers by venipuncture were centrifuged at $1,000 \times g$ for 15 min and plasma and buffy coat removed by aspiration. These cells were then washed three times with 0.01 M Na phosphate, 0.15 M NaCl, 5.6 mM glucose at pH 7.4 and 4 °C. Uptake of $^{86}Rb^+$ was measured as previously described (Smith *et al.*, 1972). Cells were incubated with $^{86}Rb^+$ for two hours. Active uptake was defined as the portion of total uptake that was inhibitable by 10^{-4} M ouabain. Duplicate samples reported in Table 2 agreed within 8% or better.

Human Red Cell Ghost $^{22}Na^+$ Efflux Studies

Lysis and resealing of human red cells for measurement of $^{22}Na^+$ efflux was carried out exactly as described by Askari (1971), except that hypotonic lysis was accomplished by dilution of one volume of packed cells with 9 volumes of hemolysis solution containing 12 mM NaCl, 2 mM Na_2ATP, and 3 mM $MgCl_2$. In experiments in which specific and control γ globulins were intended to have access to the inside of resealed ghosts, lysis was carried out in the presence of 5 or 20 mg/ml of γ globulin from the source indicated. Type II ghosts, in which the Na^+ pump was driven by ATP in the absence of substrate metabolism, were used immediately after preparation in all studies. $(Na^+ + K^+)$-ATPase-mediated $^{22}Na^+$ efflux was calculated as the difference between rates of efflux in the absence and presence of 10^{-4} M ouabain.

In experiments designed to document the uptake of antibody into the interior of lysed and resealed red cells, γ globulin preparations were labeled with ^{125}I by the method of Hunter and Greenwood (1962) and tracer amounts added to γ globulin preparations before lysis and resealing. Estimates of intracellular volume were based on packed cell volume after centrifugation of resealed ghost suspensions in microhematocrit tubes. After lysis, resealing, and washing three times with isotonic saline at 0–4 °C, ^{125}I counts showed a linear incorporation of γ globulin by resealed ghosts over the concentration range studied. Final intracellular γ globulin concentrations were within 15% of values initially present in the medium at the time of hypotonic lysis. Lysis in the presence of control or specific γ globulin preparations did not affect the amount of $^{22}Na^+$ trapped during the resealing process, as judged by count rates measured at the beginning of efflux experiments.

Preparation and Study of Inside-Out and Right-Side-Out Red Cell Membrane Vesicles

Everted and normally oriented vesicles of human red cell membranes were prepared and separated by the method of Steck, Weinstein, Straus and Wallach (1970). Linear 2–17% (1.02–1.07 g/cm^3) dextran T-110 gradients were run for 16 hr using an SW-41 rotor at 30,000 RPM in a Spinco Model L5-65 preparative ultracentrifuge. The vesicles were harvested and washed twice with 150 mM NaCl, 5 mM Na phosphate, pH 8.0, before use.

$(Na^+ + K^+)$-ATPase activities in the presence of control and specific antibodies were measured using γ-^{32}P-ATP in media containing 40 mM NaCl, 20 mM KCl, 1.25 mM $MgCl_2$, 0.25 mM H_4EDTA, 10 mM Tris Cl (adjusted to pH 7.4), 1 mM unlabeled ATP, and about 10^{-7} M γ-^{32}P-ATP. Basal ATPase activity in the absence of Na^+ and K^+ was determined by the use of 60 mM choline Cl in place of NaCl and KCl. The remainder of the assay was carried out as previously described (Smith *et al.*, 1972). $(Na^+ + K^+)$-ATPase was defined as the increment in activity resulting from addition of NaCl and KCl to incubation media. Gamma globulin concentrations of 2 mg/ml were used in these experiments.

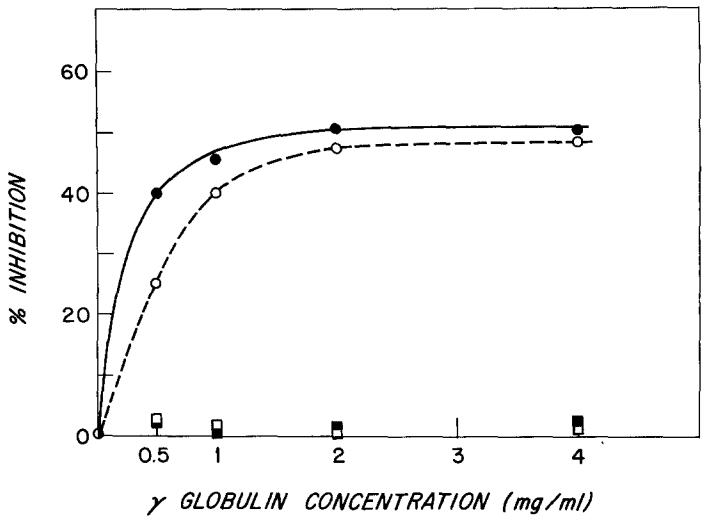

Fig. 1. Inhibition of $(Na^+ + K^+)$-ATPase from canine renal medulla by purified γ globulins from 12-week postimmunization bleedings of rabbits A_1 (closed circles) and A_2 (open circles). Antibody globulins from preimmune bleedings of rabbits A_1 and A_2, as well as 12-week postimmunization bleedings of A_3 and A_4, produced less than 6% inhibition at all concentrations tested (up to 4.0 mg/ml). Mg^{++}-ATPase (ouabain-insensitive) activity was not significantly inhibited by γ globulin fractions from rabbits A_1 (closed squares) and A_2 (open squares). All values shown are means of duplicate determinations which agreed within 10% (average range 6%)

Results

Antibody Inhibition of Canine Renal Medullary $(Na^+ + K^+)$-ATPase

Antibody responses of rabbits challenged with purified $(Na^+ + K^+)$-ATPase were first assessed by measurement of the ability of γ globulin fractions of sera from these animals to inhibit ATPase activity of dog kidney enzyme preparations. As shown in Fig. 1, concentration-related inhibition of $(Na^+ + K^+)$-ATPase activity occurred in the presence of purified γ globulin fractions from 12-week bleedings of both rabbit A_1 and A_2. For these bleedings, inhibition plateaued at about 50% of complete inhibition, and further increases in γ globulin concentration did not bring about further inhibition of enzymatic activity. In contrast, neither control (preimmunization) γ globulins nor γ globulin fractions from rabbits challenged with red cell ghost material had a significant effect on $(Na^+ + K^+)$-ATPase activity. Residual activity in the presence

of these nonspecific fractions was 95% or greater at all concentrations tested up to 4.0 mg/ml.

Inhibition of ATPase activity by specific antibody was confined to the ouabain-inhibitable fraction of total enzyme activity. Fig. 1 also summarizes experiments showing the absence of significant inhibition by γ globulins from rabbits A_1 and A_2 of Mg^{++}-ATPase activity measured in the presence of 10^{-4} M ouabain.

During the primary immune response, γ globulin concentrations of 2 mg/ml in the incubation mixture produced maximum inhibition of $(Na^+ + K^+)$-ATPase activity at the time of the 12-week bleeding. No significant inhibition was observed with gamma globulin fractions from bleedings of control rabbits at any time during the course of immunization. After booster injections at 20, 24, and 28 weeks, antibody globulins from the 32-week bleeding of rabbit A_1 produced inhibition averaging 68% at concentrations of 2 mg/ml in the assay medium. Concentrations up to 5 mg/ml caused no further increment in $(Na^+ + K^+)$-ATPase inhibition. Gamma globulin from the 32-week bleeding from rabbit A_2 caused inhibition of 65% of ouabain-inhibitable ATPase activity.

The kinetics of inhibition of $(Na^+ + K^+)$-ATPase activity in the presence of 2 mg/ml of γ globulin from the 12-week bleeding of rabbit A_1 were studied by a linked enzyme assay in which ATP cleavage was coupled through pyruvate kinase and lactate dehydrogenase to an NADH to NAD^+ transition continuously recorded at 340 nm. The onset of inhibition of $(Na^+ + K^+)$-ATPase activity was quite rapid, being evident within the 1 to 2 min required for the cuvette to be returned to the sample chamber of the spectrophotometer and a reliable slope determined after addition of the γ globulin fraction. Final steady-state inhibition occurred within 3 to 4 min. Lack of enzyme inhibition by control (rabbits A_3 and A_4) and preimmunization antiserum was also confirmed using the linked enzyme assay. The rapidity of onset of $(Na^+ + K^+)$-ATPase inhibition suggested that the mechanism of inhibition was not simply due to effective removal of enzyme from the reaction mixture by formation of an immune precipitate. This impression was confirmed by experiments in which Fab fragments of specific and control gamma globulin fractions were tested for inhibitory activity. Monovalent fragments of specific antibodies from rabbit A_1 formed by papain digestion produced 62% inhibition of $(Na^+ + K^+)$-ATPase activity, comparable to the degree of inhibition caused by the intact gamma globulin fraction. Fab fragments, like the intact parent γ globulin fraction, caused no alteration in Mg^{++}-ATPase activity over the range of concentrations tested.

Table 1. Effects of 2.0 mg/ml concentrations of γ globulin or Fab fragment fractions on $(Na^+ + K^+)$- and Mg^{++}-ATPase from various sources[a]

Enzyme source	Antibody preparation	n	$(Na^+ + K^+)$-ATPase	Mg^{++}-ATPase
Canine renal medulla	None	7	100 ± 4	100 ± 3
	$A_1 + A_2$	7	33 ± 4[b]	101 ± 5[c]
	$A_3 + A_4$	7	98 ± 6[c]	96 ± 6[c]
	Fab (A_1)	4	38 ± 6	100 ± 4[c]
Canine myocardium (microsomal)	None	6	100 ± 5	100 ± 5
	$A_1 + A_2$	6	40 ± 6[b]	94 ± 8[c]
	$A_3 + A_4$	6	95 ± 7[c]	98 ± 5[c]
Canine myocardium (Lubrol-solubilized)	None	4	100 ± 6	100 ± 5
	A_1	4	50 ± 7[b]	95 ± 7[c]
	A_3	4	97 ± 4[b]	105 ± 6[c]
Calf brain	None	4	100 ± 3	100 ± 5
	A_1	4	73 ± 8[b]	102 ± 6[c]
	A_3	4	102 ± 5[c]	99 ± 4[c]
Human red cell membrane	None	6	100 ± 5	102 ± 7
	$A_1 + A_2$	6	2 ± 3[b]	94 ± 9[c]
	$A_3 + A_4$	6	99 ± 6[c]	97 ± 5[c]

[a] Values expressed as % of enzyme activities determined in the absence of added γ globulin, ± 1 standard deviation.
[b] $P < 0.01$ compared with control ($A_3 + A_4$) γ globulins.
[c] Not significantly different ($p > 0.05$) from value in absence of added γ globulin.

Antibody Inhibition of $(Na^+ + K^+)$-ATPase from Other Sources

Results of studies of antibody interactions with $(Na^+ + K^+)$-ATPase and Mg^{++}-ATPase from various sources are summarized in Table 1. Seven determinations of inhibition of canine renal medullary $(Na^+ + K^+)$-ATPase by concentrations of specific antibody from the 32-week bleedings of rabbit A_1 and A_2 producing maximal inhibition yielded a mean inhibition of 67%. At maximum inhibitory concentrations, the effects of gamma globulins from rabbits A_1 and A_2 were similar.

Antibodies from rabbits A_1 and A_2 also inhibited $(Na^+ + K^+)$-ATPase (but not Mg^{++}-ATPase) from canine myocardial microsomal preparations (Table 1). Solubilization of $(Na^+ + K^+)$-ATPase activity from canine heart with Lubrol-WX had relatively little effect on the degree of inhibition by antibody with maximal inhibition averaging 50% compared with 60% for the microsomal form of the enzyme.

Calf brain $(Na^+ + K^+)$-ATPase was less strongly inhibited by anti-

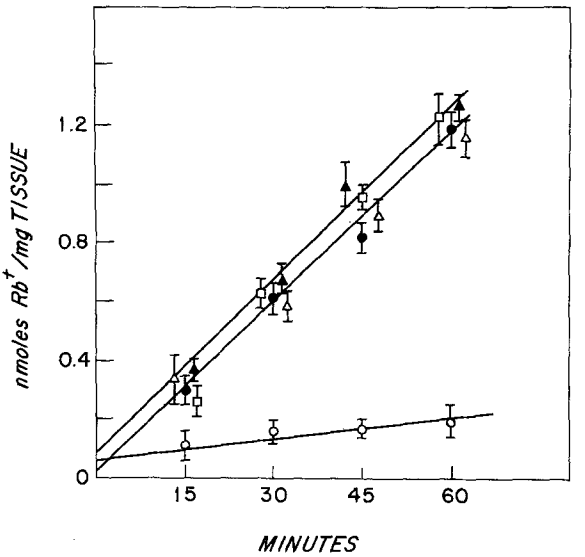

Fig. 2. $^{86}Rb^+$ uptake by slices of canine renal medulla. Both passive uptake in the presence of 10^{-4} M ouabain (open circles) and active uptake were linear over the initial 60 min of incubation. The rates of uptake in the presence of 2 mg/ml γ globulin from rabbits A_1 (closed triangles) and A_2 (closed circles) were not significantly different from mean values in the presence of control γ globulin (open triangles), nor from samples incubated with buffer alone (open squares). Mean values are plotted with bars depicting ± 1 SD. Control γ globulin values (open triangles) are means of four slices, two of which were incubated with rabbit A_2 preimmune γ globulin and two with rabbit A_3 γ globulin. Lines were determined by least-squares linear regression analysis of data obtained in the presence of postimmune γ globulin from rabbits A_1 and A_2

bodies from rabbits A_1 and A_2; maximal inhibition averaged only 27% but was statistically significant.

In contrast to the partial degrees of inhibition exerted on dog kidney, dog heart, and calf brain $(Na^+ + K^+)$-ATPases, the activity of human red cell ghost $(Na^+ + K^+)$-ATPase was virtually completely eliminated at concentrations of 2 mg/ml of rabbit A_1 or A_2 γ globulin (32-week bleedings). Thus, antibodies against canine renal medulla $(Na^+ + K^+)$-ATPase caused inhibition of this enzyme system as obtained from other organs and other species, consistent with the existence of similar or identical antigenic determinants in preparations from these diverse sources.

Antibody Effects on Monovalent Cation Transport in Intact Cells

Canine Kidney. Slices of outer medulla from dog kidneys took up $^{86}Rb^+$ from the medium by both active (ouabain inhibitable) and passive

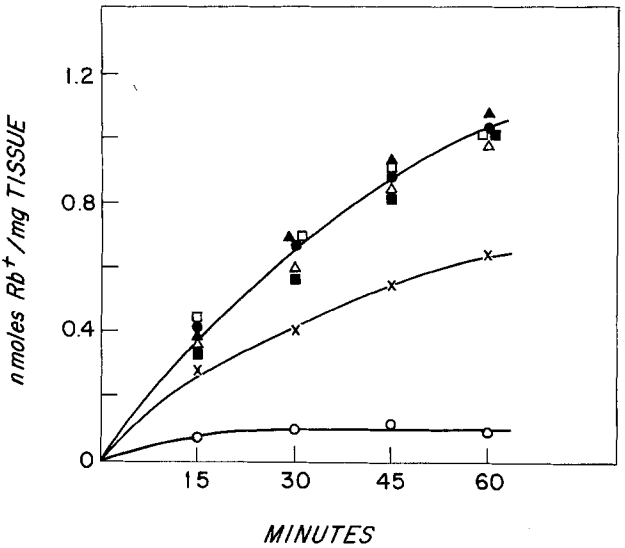

Fig. 3. $^{86}Rb^+$ uptake by guinea pig left atrial strips. Values shown are means of duplicate experiments, the average range for which was 9% of the mean. Specific γ globulin (2 mg/ml) from rabbits A_1 (closed circles) and A_2 (open triangles) did not produce results significantly different from buffer alone (open squares), preimmune γ globulin from rabbit A_2 (closed squares), or γ globulin from rabbit A_3 (closed triangles). Pronounced inhibition of uptake was evident in the presence of 10^{-7} M ouabain (x). Virtually complete inhibition of active uptake was evident within 15 min in the presence of 10^{-4} M ouabain

mechanisms. Fig. 2 summarizes the results of experiments in which slices were incubated in the presence of 2 mg/ml concentrations of specific or control antibody. Gamma globulin fractions from both rabbit A_1 and A_2 which produced 65–70% inhibition of $(Na^+ + K^+)$-ATPase prepared from canine kidney had no detectable effect on either active or passive components of $^{86}Rb^+$ uptake by these slices.

Guinea Pig Left Atrial Strips. As shown in Fig. 3, no inhibition of $^{86}Rb^+$ uptake was observed in the presence of 2 mg/ml of γ globulin from rabbit A_1 or A_2. Uptake values were not significantly different from those observed in the presence of control or preimmune γ globulin fractions. Inhibition of $^{86}Rb^+$ uptake was readily demonstrable in the presence of 10^{-7} as well as 10^{-4} M ouabain.

Human Red Blood Cells. Human erythrocytes were also used as a model to determine the effects of antibodies specific for $(Na^+ + K^+)$-ATPase on monovalent cation transport. In agreement with the results of studies with canine kidney slices and guinea pig left atrial strips, no effects of γ globulin preparations from rabbit A_1 or A_2 on intact red cell $^{86}Rb^+$ transport were observed. Cells incubated with 2 mg/ml γ globulin from 32-week bleedings of rabbits A_1 and A_2 showed a mean

ouabain-inhibitable $^{86}Rb^+$ uptake of 53.5 nmoles per ml packed cells per hour, essentially identical to values of 54.0 in the absence of any γ globulin and 52.3 in the presence of control γ globulin preparations from preimmune bleedings of rabbits A_1 and A_2 and from 32-week bleedings of rabbits A_3 and A_4.

Thus, studies in all three intact cell systems failed to demonstrate any measurable effect of antibody on $^{86}Rb^+$ transport, despite the fact that these antibody preparations were capable of substantial inhibition of $(Na^+ + K^+)$-ATPase from canine renal medulla, canine ventricular myocardium, and human red blood cells in disrupted cell preparations (Table 1).

It is of interest that serial measurements of serum Na^+ and K^+ concentration in rabbits A_1 and A_2 at the time of each bleeding showed consistently normal Na^+ values of 138–144 and K^+ values of 3.6–4.2 mEq/liter throughout the course of immunization. There was no *in vivo* evidence of alteration of Na^+ or K^+ transport by circulating antibodies as judged by these serum electrolyte values.

Antibody Effects on $^{22}Na^+$ Transport in Lysed, Resealed Human Red Cell Ghosts

The above data indicated that the portion of the $(Na^+ + K^+)$-ATPase complex with which specific antibodies interacted to cause enzyme inhibition was not accessible at the outer surface of intact cells. We therefore undertook studies to test the hypothesis that the antigenic determinants with which the antibodies interact to inhibit $(Na^+ + K^+)$-ATPase turnover are at the inner cell surface. Lysis and resealing of red blood cells according to the method of Askari (1971) permitted introduction of antibody as well as ATP and $^{22}Na^+$ to the interior of red cell ghosts. As described in Materials and Methods, initial experiments showed that γ globulin was in fact being trapped in the interior of the resealed ghost preparations in concentrations roughly equivalent to the concentration of γ globulin present at the time of hypotonic lysis.

First order kinetics were observed for Na^+ efflux over the initial 70% of the process. The presence of 10^{-4} M ouabain reduced the rate constant for $^{22}Na^+$ efflux from 0.66 to 0.11 hr^{-1}, in agreement with the observations of Askari and Rao (1970). As summarized in Table 2, control γ globulin from rabbit A_3 and preimmune γ globulin from rabbit A_1 also caused reductions in the rate of Na^+ efflux from resealed red cell ghosts. At 0.4 mg/ml of γ globulin, the efflux rate constant was

Table 2. $(Na^+ + K^+)$-ATPase-mediated $^{22}Na^+$ efflux from resealed human red blood cell ghosts

γ Globulin added	$(Na^+ + K^+)$-ATPase-mediated $^{22}Na^+$ efflux rate[a]
None	100
Rabbit A_1 (32 weeks)	
0.4 mg/ml	42
2.0 mg/ml	0
Rabbit A_1 (preimmunization)	
0.4 mg/ml	78
2.0 mg/ml	55
Rabbit A_3 (32 weeks)	
0.4 mg/ml	82
2.0 mg/ml	56

[a] Expressed as percent of ouabain-sensitive efflux rate in the absence of added γ globulin. Values given are from least-squares plots of log % residual intracellular $^{22}Na^+$ *vs.* time from 0 to 150 min, after correction for the residual $^{22}Na^+$ efflux rate observed under conditions identical except for the presence of 10^{-4} M ouabain. Correlation coefficients were 0.95 or better for all plots.

reduced by 18–22% and at 2 mg/ml by 44–45%. Substantially greater inhibition of Na^+ efflux was observed in the presence of antibodies from rabbit A_1 capable of inhibiting $(Na^+ + K^+)$-ATPase, however. Fifty-eight percent inhibition was observed at a concentration of 0.4 mg/ml in the initial lysis medium, and at 2.0 mg/ml $(Na^+ + K^+)$-ATPase-mediated Na^+ efflux was totally inhibited. The ouabain-insensitive $^{22}Na^+$ efflux rate increased slightly (mean 9%) in ghosts resealed in the presence of either control or specific γ globulin. This difference was not statistically significant. No significant inhibition of Na^+ efflux was observed with either control or specific γ globulin fractions at concentrations of 0.4 or 2.0 mg/ml when added subsequent to the completion of lysis and resealing of red cell membranes, consistent with the above-described lack of effect of control or specific antibody on active $^{86}Rb^+$ uptake by intact human red cells. Thus, $^{22}Na^+$ efflux mediated by $(Na^+ + K^+)$-ATPase was markedly inhibited in lysed, resealed human red cell ghosts by antibodies specific for canine renal medullary $(Na^+ + K^+)$-ATPase, but only if the antibody had access to the inner surface of these membranes. The reason or reasons for partial inhibition of Na^+ efflux by nonspecific γ globulin fractions in lysed, resealed red cell ghost preparations (albeit to a considerably lesser extent than specific antibodies), is not apparent. We cannot exclude with certainty the possibility that antisera cause preferential resealing of ghosts that have an inherently lower rate of $^{22}Na^+$ efflux.

Table 3. Effects of γ globulin fractions on $(Na^+ + K^+)$-ATPase activity in right-side-out (RO) and inside-out (IO) red cell membrane vesicles[a]

	Mg^{++}-ATPase[b]		$(Na^+ + K^+)$-ATPase[c]	
	RO	IO	RO	IO
No γ globulin	100±6	100±4	100±7	100±6
A_2 (32 weeks)	112±9	106±10	73±5	−2±4
A_3 (32 weeks)	90±6	96±5	92±10	94±8
A_2 (preimmunization)	97±5	101±7	88±8	96±6

[a] Values stated as percent±standard error of the mean ($n=4$), relative to values in the absence of γ globulin. All γ globulin concentrations were 2 mg/ml in incubation media.
[b] ATPase activity with NaCl and KCl replaced by 60 mM choline Cl.
[c] Increment in activity in presence of 40 mM NaCl, 20 mM KCl.

Antibody Effects on $(Na^+ + K^+)$-ATPase Activity of Inside-Out and Right-Side-Out Red Cell Membrane Vesicles

In view of the problems just described in interpretation of $^{22}Na^+$ efflux experiments with lysed, resealed red cell ghosts, we sought additional means to determine the locus of antibody interaction with $(Na^+ + K^+)$-ATPase which resulted in enzyme inhibition. $(Na^+ + K^+)$-ATPase activities of two ghost preparations used in these studies, prior to vesiculation and density gradient centrifugation, were 580 and 850 pmoles/mg protein/min. Inside-out vesicle fractions gave values similar to those from right-side-out vesicles, with a range from 340 to 530 pmoles/mg protein/min. Data in Table 3 are expressed as percentages of values in the absence of γ globulin, with the activity of each preparation in the absence of γ globulin being taken as 100%. $(Na^+ + K^+)$-ATPase activity of right-side-out red cell ghost vesicles was reduced to a minor extent by control (A_3) and preimmune γ globulin fractions, with 88–92% of activity remaining in the presence of 2 mg/ml of nonspecific antibody. Gamma globulin from the 32-week bleeding of rabbit A_2 caused 27% inhibition of $(Na^+ + K^+)$-ATPase activity in right-side-out vesicles. In marked contrast, $(Na^+ + K^+)$-ATPase activity of inside-out vesicles was completely inhibited at the same concentration of specific antibody from rabbit A_2. Control γ globulin fractions caused no significant inhibition of the inside-out vesicle fraction. As in the experiments summarized in Table 1, no significant effect on the nonouabain inhibitable fraction of ATPase activity was observed in the presence of control or specific antibody. These results support the conclusion that the antigenic deter-

minant(s) with which specific antibodies interact to inhibit $(Na^+ + K^+)$-ATPase activity and monovalent cation active transport are accessible only from the inner surface of the cell membrane.

Discussion

Recent methodologic advances, including the development of SDS-polyacrylamide gel electrophoresis and selective labeling of proteins exposed at the outer cell surface, have spurred progress in localization of components of biomembranes (Bretscher 1973*b*; Singer, 1974; Steck, 1974). Several laboratories have recently made use of antibodies in studies of monovalent cation transport and of its presumed molecular mediator, $(Na^+ + K^+)$-ATPase. Averdunk, Günther, Dorn and Zimmermann (1969) reported that rabbit antisera from animals challenged with a crude human erythrocyte membrane preparation would inhibit $(Na^+ + K^+)$-ATPase and Mg^{++}-ATPase in such preparations. Lauf (1975) has recently reviewed studies of iso-antibodies against the erythrocyte L antigen of sheep and goats, which stimulate active monovalent cation transport in low potassium (LK) red cells, and enhance $(Na^+ + K^+)$-ATPase activity in LK red cell membranes. There is no evidence from these studies with LK cells, however, that direct interaction of antibody with $(Na^+ + K^+)$-ATPase *per se* is taking place.

More recently, Askari and Rao (1972) reported studies with an antiserum directed against the $(Na^+ + K^+)$-ATPase complex of rat brain. A partially purified enzyme preparation was used as an antigen, and antibodies obtained against this material were studied for their effects on reactions catalyzed by $(Na^+ + K^+)$-ATPase from the same source used as antigen. The interaction of the antibody with the $(Na^+ + K^+)$-ATPase was found to cause almost complete inhibition of $(Na^+ + K^+)$-ATPase activity, but had little or no effect on the K^+-dependent *p*-nitrophenylphosphatase activity. Na^+-dependent phosphorylation of the complex by ATP was partially blocked in the presence of the antibody, but the K^+-dependent breakdown of the phosphoprotein was not affected in a way which could be discerned by the methods used. Subsequent studies by Askari (1974) have shown an ability of some anti-$(Na^+ + K^+)$-ATPase antisera to inhibit the K^+-dependent phosphatase partial reaction, but to a considerably lesser extent than $(Na^+ + K^+)$-ATPase activity was inhibited.

Kyte (1974) has studied antibodies against $(Na^+ + K^+)$-ATPase prepared from canine renal medulla by the same technique used in the studies reported here. These antibodies were found to bind to the native enzyme only at sites on one or the other of the two polypeptide subunits present in the preparation used as antigen. Ultrastructural studies using ferritin-conjugated γ globulins indicated that antigenic sites on the larger subunit were located only on the inner surface of the plasma membrane. Interestingly, only minimal $(Na^+ + K^+)$-ATPase inhibition was observed when catalytic subunit-specific γ globulins were bound to the enzyme at saturating concentrations. It is thus apparent, as might be expected from the known heterogeneity of immune responses to a given antigen among individual animals, that the effects of antibodies to $(Na^+ + K^+)$-ATPase may vary substantially according to the response of the individual experimental animal challenged. Heterogeneity of the immune response even within an individual animal is supported by the studies of McCans *et al.*, (1974), who have presented evidence that an antiserum against a purified $(Na^+ + K^+)$-ATPase from canine kidney could be fractionated into two globulin components, one of which inhibited catalytic activity of the enzyme and the other of which inhibited cardiac glycoside binding. It is of interest that the specific enzyme-inhibitory globulin fraction studied by McCans *et al.* (1974) produced a maximal inhibition of 80% of $(Na^+ + K^+)$-ATPase activity, similar to the values obtained for maximal inhibition in the experiments reported here.

Jørgensen and Glynn and their colleagues have studied the effects of antibodies to $(Na^+ + K^+)$-ATPase from a pig kidney source (Jørgensen *et al.*, 1973; Glynn *et al.*, 1974). Antisera from rabbits immunized with a purified preparation of $(Na^+ + K^+)$-ATPase from pig kidney outer medulla inhibited $(Na^+ + K^+)$-ATPase in several tissues studied, including rabbit kidney and ox brain as well as the preparation from pig kidney used as antigen. These authors found that when the antiserum was incorporated into human red cell ghosts at the time of hemolysis, the ouabain-sensitive efflux of Na^+ from the ghosts was completely abolished. These effects on cation pumping were observed only when the immune serum had access to the inner surface of the red cell membrane. Analogous results were observed by Askari (1974) with lysed, resealed rat red cell ghosts, although the antibody to rat brain $(Na^+ + K^+)$-ATPase studied by Askari showed no evident crossreaction with human red cell $(Na^+ + K^+)$-ATPase. The present experiments confirm and extend these results by demonstrating interaction of antibodies raised against dog kidney $(Na^+ + K^+)$-ATPase with antigenic deter-

minants of $(Na^+ + K^+)$-ATPase from such diverse sources as dog heart, calf brain, and human erythrocytes (Table 1). Enzyme from eel electroplax, prepared by minor modification of the method of Bauman, Changeux and Benda (1969) also showed 50% inhibition by $(Na^+ + K^+)$-ATPase-specific antiserum from rabbit A_1 (data not shown). Purified γ globulin fractions were not tested for inhibition of the eel electroplax enzyme. The differing degrees of inhibition observed in various preparations are not explained by these experiments, but raise the question of differing exposure of relevant antigenic determinants due to formation of closed vesicles during enzyme preparations. Since all inhibition values were determined at antibody concentrations beyond which no further inhibition occurred, it is unlikely that the observed differences are due to differing total numbers of enzyme complex copies in assay tubes. Higher density of enzymatic units in certain membrane preparations could conceivably result in protection of some units by steric hinderance of further interaction by adjacent antibody-ATPase complexes. This sort of phenomenon could account for the observation that the greatest relative degrees of inhibition occurred in red cell membrane preparations, which are known to have a comparatively low density of $(Na^+ + K^+)$-ATPase sites (Hoffman, 1969). It would not, however, account for the observation that the canine heart and kidney enzymes were inhibited to a similar extent despite a 40-fold difference in the specific activities of the preparations tested, nor would it account for the similar plateau level of inhibition seen with intact γ globulin and with Fab fragments.

Several lines of evidence from the studies reported here support the conclusion that specific antibody interactions were responsible for the observed effects on $(Na^+ + K^+)$-ATPase activity and monovalent cation transport. Studies were carried out on γ globulin fractions which had undergone extensive purification by ammonium sulfate precipitation and DEAE cellulose chromatography. Nonspecific effects from other major serum protein fractions were thus eliminated. Exhaustive dialysis rendered effects from small, dialyzable components unlikely. All experiments were controlled by the use of identically treated γ globulin fractions from preimmune bleedings of rabbits subsequently immunized (A_1 and A_2) as well as fractions from rabbits that had undergone an identical immunization schedule, but with a different antigen. These effects of specific antibody globulins were confined to the Na^+ and K^+-activated, ouabain-inhibited component of total ATPase activity. In no instance was significant alteration of Mg^{++}-ATPase activity observed.

In agreement with the findings of Jørgensen *et al.* (1973), exposure

of the inner surface of red cell ghost membranes to antibody globulins by lysis and resealing of red cells in the presence of specific or control γ globulins resulted in inhibition of $(Na^+ + K^+)$-ATPase-mediated $^{22}Na^+$ efflux. Only that portion of $^{22}Na^+$ efflux which could be inhibited by 10^{-4} M ouabain was affected and then only when antibody had access to the inner cell surface. Although considerably greater degrees of inhibition occurred in the presence of specific γ globulins, significant inhibition was uniformly found with nonspecific γ globulin fractions as well. In addition to γ globulins from rabbits A_3 and A_4 and preimmune bleedings from rabbits A_1 and A_2, a total of four bleedings from other individual rabbits not immunized with $(Na^+ + K^+)$-ATPase were studied. All showed comparable degrees of ouabain-sensitive $^{22}Na^+$ efflux inhibition. Because of this problem of apparent nonspecific interaction between purified rabbit γ globulin and active $^{22}Na^+$ transport in human red cell membranes, we sought additional evidence regarding the localization (sidedness) of specific antibody interaction with the monovalent cation transport complex. Using inside-out (everted) and right-side-out red cell membrane vesicles, complete inhibition of $(Na^+ + K^+)$-ATPase activity by specific antibody globulins was observed in the inside-out vesicle population, while only 27% inhibition was found in right-side-out vesicles. This latter degree of inhibition is probably due, in large part, to contamination of the predominantly right-side-out vesicle fraction by inside-out vesicles (Steck *et al.*, 1970; Kant & Steck, 1972). There was no significant change in $(Na^+ + K^+)$-ATPase activity values of right-side-out vesicle fractions by either specific or control antibody preparations.

Studies of antibody effects on monovalent cation transport in intact guinea pig left atrial strips [originally performed to detemine whether the hypothesis could be tested that specific inhibition of monovalent cation transport might produce a positive inotropic effect on the myocardium, as has been postulated in the case of cardiac glycosides (Langer, 1968)] showed no inhibitory effect on active $^{86}Rb^+$ uptake. This finding was confirmed in canine renal medulla slices and intact human red cells as well. The observed absence of effects in any of these systems indicates that determinants with which specific antibodies interact to produce inhibition of $(Na^+ + K^+)$-ATPase activity and monovalent cation active transport are not readily accessible to antibodies at the outer cell surface.

Taken together with the data shown in Tables 1 and 2, these results support previous inferences that $(Na^+ + K^+)$-ATPase is readily accessible only at the inner surface of plasma membranes. One cannot exclude, on the basis of these data, the possibility that components of the monova-

lent cation transport complex are present at the outer cell surface but fail to elicit an antibody response, or that antibodies are formed to externally exposed antigens but fail to affect $(Na^+ + K^+)$-ATPase activity or monovalent cation transport (Kyte, 1974). As suggested by Jørgensen *et al.* (1973), it is also possible that antibodies formed in response to antigens accessible at the outer cell surface bind to such antigens in the immunized animal and thus are not present in appreciable amounts in the serum. Evidence in favor of such a mechanism was not obtained in the present studies in that neither hemolysis nor altered plasma Na^+ or K^+ concentrations were observed in serial bleedings of immunized animals.

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