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Effects of an Antibody to a Highly Purified Na^+, K^+-ATPase from Canine Renal Medulla: Separation of the "Holoenzyme Antibody" into Catalytic and Cardiac Glycoside Receptor-Specific Components

(antibody fractionation/globulin components/digitalis receptor antibody/ conformation-specific antibodies)

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ABSTRACT An antiserum was prepared against a highly purified Na^+, K^+-adenosine triphosphatase (ATP phosphohydrolase, EC 3.6.1.3). Purification and fractionation yielded two globulin components, one of which appears specific for a digitalis glycoside receptor site or binding conformation and the other for a catalytic site or conformation.

Enzyme-specific antibodies have proven to be sensitive and powerful probes for the purpose of elucidating structural and functional characteristics of certain enzymes (1, 2). Such antibodies may produce inhibition or stimulation of enzyme activity, may alter certain physical characteristics such as heat stability, or may react with the enzyme without causing detectable changes in enzymatic activity (1).

Antisera to Na^+, K^+-ATPase (ATP phosphohydrolase, EC 3.6.1.3) have been produced and their effects upon enzyme function have recently been published (3–7). In the studies thus far reported, antiserum (3, 5, 6) or an ammonium sulfate-precipitated globulin fraction (4, 7) was employed. Interpretation of the effects of such antibodies upon enzyme function is complex, since antisera against protein macromolecules ordinarily contain numerous antibody populations specific for various portions (antigenic determinants) of the molecule. The observed effects, therefore, are likely to be the result of several antibodies with different or even opposing effects upon the enzyme.

The present report describes a procedure whereby antiserum to Na^+,K^+-ATPase is purified and separated into two fractions containing antibodies with different effects upon catalytic activity of the enzyme and binding of [^{3}H]ouabain to the enzyme.

MATERIALS AND METHODS

Production of antiserum

White, New Zealand rabbits were immunized with highly purified Na^+,K^+-ATPase prepared from canine renal outer medulla by the method of Lane *et al.* (8). This enzyme preparation consists of two polypeptides in a molar ratio of 1.0 and contains "supporting" lipids. Enzyme (antigen) was suspended in saline and mixed with an equal volume of Freund's (complete) adjuvant to a final antigen concentration of 1 mg/ml. The rabbits received 0.6 ml of this suspension subcutaneously into the toe-pads (0.2 ml in two rear toe-pads, 0.1 ml in two front toe-pads) weekly for 3 weeks, followed by 0.2 ml intramuscularly into each haunch weekly for 4 weeks. Bleeding was commenced 1 week after the haunch injections and was carried out bimonthly. Booster injections of antigen (0.3 ml into each haunch) were administered on the weeks between bleedings.

Purification and fractionation of antiserum

Ammonium Sulfate [$(NH_4)_2SO_4$] *Precipitation.* Precipitation of globulin from antiserum was carried out by the dropwise addition, with constant stirring, of a volume of saturated $(NH_4)_2SO_4$ (pH 7.4) equal to 50% of the volume of serum (9). The precipitate was collected by centrifugation at 10,000 × *g* for 30 min and redissolved in a volume of deionized water equal to 50% of the original volume of serum. The precipitation and resolubilization cycle was repeated three times. The globulin fraction was then dialyzed against 150 mM Tris·HCl buffer (pH 7.4) at a rate of 40 volumes/12 hr with eight changes of the dialysate and a total dialysis period of 96 hr. These conditions of dialysis were found adequate for the removal of residual $(NH_4)_2SO_4$, which was monitored by the addition of saturated barium chloride to aliquots of the dialysate.

Absorption of Globulin Fraction with Enzyme. A concentrated suspension of Na^+,K^+-ATPase (10–15 mg/ml) was added to the globulin obtained by $(NH_4)_2SO_4$ precipitation. Sufficient enzyme was added to bind all specific antibody (1–2 mg of enzyme per 5 mg of globulin). Enzyme and globulin were incubated at 37° for 30 min in 30 mM histidine buffer (pH 7.4) containing 5 mM $MgCl_2$, 100 mM NaCl, and 10 mM KCl. The mixture was allowed to stand at 4° overnight and was then centrifuged at 40,000 × *g* at 4° for 30 min. The pellet was washed by resuspension in a small volume of deionized water [or a solution containing 30 mM histidine, 5 mM $MgCl_2$, 100 mM NaCl, and 10 mM KCl (pH 7.4)] followed by recentrifugation. Bound protein was then eluted from enzyme by suspending the complex in 10 mM glycine–140 mM NaCl (pH 2.8) at 20°. The volume of elution buffer added was equal to 50% of the original volume of serum. After 1 hr the enzyme was removed by centrifugation at

Abbreviations: anti-cat, anticatalytic antibody; anti-DR, anti-digitalis receptor antibody; P_i, inorganic phosphate.

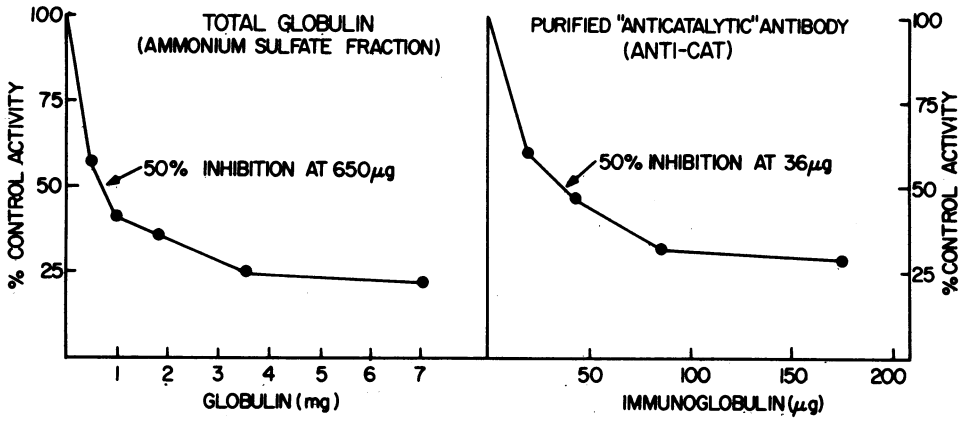

FIG. 1. Comparison of the dose-responses to the globulin fraction obtained by $(NH_4)_2SO_4$ precipitation of antiserum and to purified anticatalytic antibody. The conditions of the assay are described in the *Materials and Methods* section. In these studies, 2.5 μg of enzyme was assayed by the linked-enzyme method (10).

40,000 × *g* and the supernatant, containing the Na^+,K^+-ATPase antibody, was dialyzed against 150 mM Tris·HCl buffer (pH 7.4) at a rate of 40 volumes/12 hr with four changes of the dialysate. The utility of this fractionation step resides in the insolubility of the Na^+,K^+-ATPase preparation, which is a protein–lipid complex, in aqueous solutions. The protein eluted from the enzyme (the purified Na^+,K^+-ATPase antibodies) appeared to contain no protein moieties other than immunoglobulin, when examined by immunoelectrophoresis employing goat antiserum to whole rabbit serum.

Fractionation of Na^+,K^+-ATPase Antibody with Enzyme–Ouabain Complex. For formation of the enzyme–ouabain complex, an amount of enzyme exactly equal to that used in the preceding purification step was incubated with 1 mM [^{3}H]ouabain in the presence of 2.5 mM $MgCl_2$ and 2.5 mM P_i. Incubation was carried out for 30 min at 37° to ensure maximal [^{3}H]ouabain binding. Labeled ouabain was used in order to facilitate detection of ouabain in the final antibody fractions (see below).

At the completion of [^{3}H]ouabain binding to the enzyme, the antibody which had been purified by elution from non-ouabain treated Na^+,K^+-ATPase was added to the reaction vessel containing the enzyme–[^{3}H]ouabain complex. The ratio of the enzyme–[^{3}H]ouabain complex to antibody was 3–7 mg of enzyme protein per mg of antibody protein. Histidine buffer (pH 7.4), $MgCl_2$, NaCl, and KCl were added to give final concentrations of 30 mM, 5 mM, 100 mM, and 10 mM, respectively (identical to the ligand conditions of the previous purification step). Incubation of antibody and enzyme–[^{3}H]ouabain complex was carried out for 30 min at 37° and the mixture was then allowed to stand overnight at 4°. The enzyme–[^{3}H]ouabain complex and bound antibody were separated from unbound antibody by centrifugation at 40,000 × *g* at 4° for 30 min. The supernatant containing the unbound antibody was decanted and saved. The pellet was washed by suspension in deionized water and recentrifugation. The bound antibody was eluted from the enzyme–[^{3}H]ouabain complex by treatment with glycine-NaCl buffer (pH 2.8) (volume of buffer added was 25% of the volume of original serum) and centrifuged as described above. The antibody which was eluted from the enzyme–[^{3}H]ouabain complex was designated as "anticatalytic antibody" (anti-cat) and that which did not bind to the complex as "anti-digitalis receptor antibody" (anti-DR) for reasons which will become apparent. Both fractions were dialyzed exhaustively against 150 mM Tris·HCl buffer (pH 7.4) until aliquots of the dialysate were free of all detectable [^{3}H]ouabain. (Generally, this required dialysis against 40 volumes/12 hr with eight changes and a total dialysis time of 96 hr.) Aliquots of the antibody fractions were also counted to verify the removal of [^{3}H]-ouabain.

Control serum was obtained from non-immunized rabbits and was fractionated by $(NH_4)_2SO_4$ precipitation. The globulin fraction was dialyzed against 150 mM Tris·HCl buffer (pH 7.4).

The anti-Na^+,K^+-ATPase activity of the serum, globulin, purified antibody, and anti-cat fractions was stable for at least 2 weeks at 4° and for at least 2 months at −15°. The anti-DR fraction had similar stability.

Assay of Na^+,K^+-ATPase activities

In the studies of the effects of antibody on Na^+,K^+-ATPase, enzyme activity was routinely determined by the spectrophotometric coupled-enzyme assay (10), but was also checked by estimation of the amount of phosphate produced (11). The assay medium contained 5 mM Na_2ATP, 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, and a suitable quantity of enzyme. In the case of the coupled-enzyme assay, Na^+,K^+-ATPase was added to the assay medium and, after a stable rate of catalysis had been established, antibody or control globulin was added. In the case of the assays for phosphate

TABLE 1. *Effect of fractions of antiserum to Na^+,K^+-ATPase on enzyme activity*

Antibody fraction	Yield from one ml of antiserum (mg protein)	Protein required for 50% inhibition of Na^+,K^+-ATPase activity (μg)
Antiserum	55.0	2100
$(NH_4)_2SO_4$ globulin	17.0	650
Purified antibody	1.0	50
Anti-cat	0.48	36
Anti-DR	0.52	No inhibition

Description of the antibody fractions and the assay of Na^+,K^+-ATPase activity is in the *Materials and Methods* section.

determination, antibody or control globulin was preincubated with enzyme for 15 min at 37° in the assay medium free of ATP. Following the preincubation, the catalytic reaction was started by the addition of 5 mM Na_2ATP.

Binding of [^{3}H]ouabain

The effects of antibody on [^{3}H]ouabain binding were studied both for binding in the presence of Mg^{++}, Na^+, and ATP and in the presence of Mg^{++} and P_i. Under both ligand conditions, antibody or control globulin was preincubated with enzyme in the presence of ligands (5 mM $MgCl_2$, 100 mM NaCl, and 5 mM Na_2ATP or 2.5 mM $MgCl_2$ and 2.5 mM P_i) in 1 ml of 50 mM imidazole buffer (pH 7.4) for 15 min at 37°. Binding was initiated by the addition of 0.5 μM [^{3}H]ouabain to the incubation medium and was terminated by the addition of 1 mM unlabeled ouabain. An aliquot of the binding mixture was then passed over a Millipore filter (0.45 μm) and, after washing with deionized water, the filter was dissolved in 10 ml of scintillation fluid and counted in a Beckman LS-200 B counter as previously described (12). Protein was determined by the method of Lowry *et al.* (13).

RESULTS AND DISCUSSION

Antiserum from rabbits immunized against Na^+,K^+-ATPase inhibited catalytic activity of the enzyme up to 80%. This effect of antiserum to Na^+,K^+-ATPase is in agreement with those reported by Averdunk *et al.* (3), Wikman-Coffelt *et al.* (5), Askari and Rao (4), Jørgensen *et al.* (6), and Smith *et al.* (7). Equal amounts of control serum from nonimmunized rabbits had no effect. When the Na^+,K^+-ATPase antibody was added to enzyme that was actively turning over (using the coupled-enzyme assay method), the inhibition produced by antibody was observed to be time-dependent, reaching a maximum within 8–10 min. The inhibitory effects of the various fractions obtained from the purification and fractionation procedures are summarized in Table 1. As judged by the amount of antiserum protein required to reduce catalytic rate by 50%, these procedures effected a 58-fold purification of the anticatalytic antibody. Fig. 1 compares the dose-responses to the globulin fraction obtained by $(NH_4)_2SO_4$ precipitation and to anti-cat. Although the quantity of anti-cat required to produce a given degree of inhibition was only a fraction of that of the globulin fraction, the maximal inhibition obtained was equivalent. Complete inhibition of catalysis was not observed under these experimental conditions.

When the antibody eluted from Na^+,K^+-ATPase was incubated with the enzyme–[^{3}H]ouabain complex, approximately one-half of the antibody protein did not bind to the complex and was recovered in the supernatant. This anti-DR fraction had little inhibitory effect upon the catalytic activity of Na^+,K^+-ATPase when incubated with enzyme under turnover conditions. However, as shown in Fig. 2, anti-DR inhibited [Mg^{++},P_i]-stimulated [^{3}H]ouabain binding to the enzyme. The antibody (anti-cat) which did bind to the enzyme–[^{3}H]ouabain complex did not inhibit [^{3}H]ouabain binding despite its inhibitory effects on catalysis. Similarly, whole antiserum, $(NH_4)_2SO_4$ precipitated globulin, and antibody eluted from the enzyme did not inhibit [^{3}H]ouabain binding.

Fig. 3 presents a comparison of the effects of anti-cat and anti-DR on [^{3}H]ouabain binding when the reaction was carried out in the presence of [Mg^{++},Na^+,ATP] or [Mg^{++},P_i] (10, 12). Certain differences were observed in the effects of the antibody fractions on binding under the two ligand conditions. Anti-DR inhibited binding under both conditions, but more so in the case of [Mg^{++},P_i]-supported binding. It should be noted that the enzyme–ouabain complex that was used to separate anti-cat and anti-DR in these experiments was formed in the presence of Mg^{++} and P_i. Secondly, anti-cat had no effect upon [Mg^{++},P_i]-induced binding but appeared to *increase* the binding supported by [Mg^{++},Na^+, ATP] above that obtained in the control. However, the level of control binding obtained with [Mg^{++},Na^+,ATP] was lower than that with [Mg^{++},P_i] and the apparent increase produced by anti-cat was only to the control level obtained in the presence of [Mg^{++},P_i]. This suggests perhaps that anti-cat acted to produce a conformation more conducive to [^{3}H]ouabain binding than was present with [Mg^{++},Na^+,ATP] as ligands and indicates that the lack of an inhibitory effect of anti-cat upon binding is not due to the inability of the antibody to bind to the enzyme under the ligand conditions described.

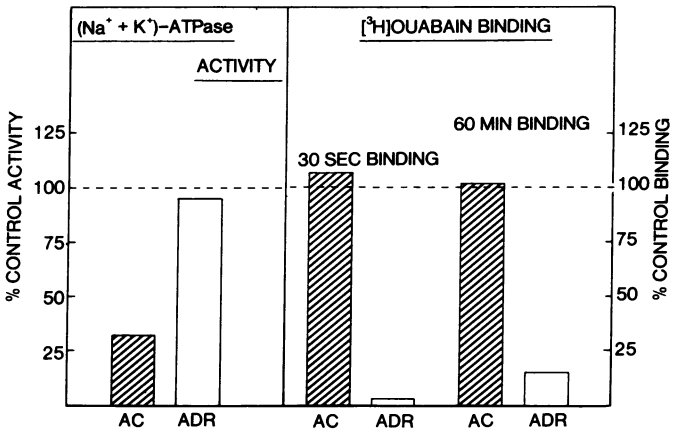

FIG. 2. Comparison of the effects of anti-cat and anti-DR on Na^+,K^+-ATPase catalytic activity and on [Mg^{++},P_i]-stimulated [^{3}H]ouabain binding. The assay conditions are described in the *Materials and Methods* section. The *left panel* demonstrates the effect of 200 μg of anti-cat or anti-DR upon the activity of 2.5 μg of enzyme. The *right panel* demonstrates the effects of 400 μg of anti-cat or anti-DR upon [^{3}H]ouabain binding to 100 μg of enzyme after 30 sec or 60 min of binding. The results are expressed as percent of the activity of enzyme treated with control globulin. AC, anti-cat; ADR, anti-DR.

The results of these studies indicate that the antiserum produced by immunization with a highly purified Na^+,K^+-ATPase contains a complex mixture of antibodies which differ with respect to the effects produced upon the enzyme. Presumably, such differences reflect the fact that individual "families" of antibodies are specific for different portions of the enzyme molecule(s) or for different conformations.

The whole antibody to Na^+,K^+-ATPase does not inhibit ouabain binding to the enzyme, but a fraction of the whole antibody does inhibit binding. This suggests that certain antibodies (anti-cat), upon complexing with the enzyme, fix it in a conformation which is consistent with ouabain binding but perhaps inconsistent with anti-DR interaction. In other words, the inability of the sera, globulin, and purified Na^+,K^+-ATPase antibody fractions to inhibit ouabain binding may result from one group of antibodies (anti-cat) preventing a second group (anti-DR) from reacting with the enzyme (14). These considerations apply to the case where the antibodies are in excess of enzyme.

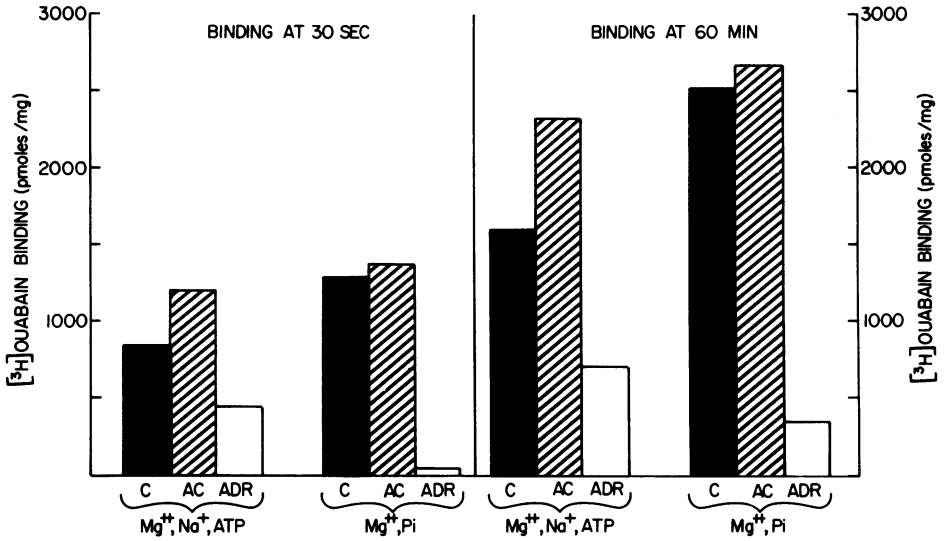

FIG. 3. Comparison of the effects of anti-DR and anti-cat on [^{3}H]ouabain binding to Na^+, Ka^+-ATPase. The assay of [^{3}H]ouabain binding is described in the *Materials and Methods* section. In these studies, 100 μg of enzyme was preincubated with 400 μg of globulin (AC, anti-cat; ADR, anti-DR; or C, control globulin) in the presence of the appropriate ligands [Mg^{++}, Na^+, ATP] or [Mg^{++}, P_i] for 15 min at 37°.

On the other hand, *fractionation* of the antibodies was carried out by incubation with the enzyme–ouabain complex where the latter was in excess. Certain species of antibody (anti-DR) which could bind to the enzyme in the presence of Mg^{++}, Na^+, and K^+ could not bind to the enzyme–ouabain complex under identical ligand conditions. Since the enzyme–ouabain complex was in excess of antibody, the interaction of ouabain with the enzyme *per se* probably prevented anti-DR from reacting. Ouabain could prevent the binding of anti-DR in one of two ways. Anti-DR may normally bind directly to the ouabain receptor, but is unable to do so when this site is already occupied by ouabain. Alternatively, anti-DR may bind to some site other than the actual ouabain receptor; the conformation of this site is so altered in the enzyme–ouabain complex that it is no longer recognizable to anti-DR. The latter possibility is perhaps more likely, but, at the present time, the former possibility cannot be discarded.

The results presented here indicate the feasibility of separating the individual antibodies by absorbing fractions onto enzyme (antigen) which has been altered such that certain structural features are no longer available. These data are also consistent with the hypothesis that one of the macromolecular determinants for digitalis binding differs from the determinants for catalytic activity (15, 16).

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