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INTERACTIONS OF LECTINS WITH ($Na^+ + K^+$)-ATPase OF EEL ELECTRIC ORGAN

ALAN C. SWANN[a], AMIRAM DANIEL[b], R. WAYNE ALBERS[a] and GEORGE J. KOVAL[a]

[a]*Laboratory of Neurochemistry and* [b]*Surgical Neurology Branch, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)*

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SUMMARY

Interaction of lectins with a detergent-solubilized ATPase from eel electric organ was studied. Concanavalin A, which binds to α-mannosides, altered the rate of enzyme migration in agar and inhibited the formation of an antigen-antibody precipitate; other lectins had no such effects. Concanavalin A similar amounts partially inhibited ($Na^+ + K^+$)-ATPase; this inhibition was reversible by α-methylglucoside. There was no corresponding effect of concanavalin A on the potassium *p*-nitrophenylphosphatase. Concanavalin A also did not interfere with ouabain binding. Thus, concanavalin A binds to an antigenic region also involved in Na^+ and/or ATP binding, but does not interact with a K^+ site.

INTRODUCTION

($Na^+ + K^+$)-ATPase (EC 3.6.1.3) is an active transport unit for Na^+ and K^+ which spans the plasma membrane [1, 2, 22], and is so well integrated into the structure of the membrane that complete isolation in an active form is difficult [3]. The ATPase is a complex protein, consisting of at least two types of polypeptide, which can be separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [4–7]. The larger polypeptide, having a molecular weight of about 100 000, is phosphorylated by ATP in the presence of Na^+; the enzyme must be in the intact form for phosphorylation to occur [7, 8]. The smaller unit, of molecular weight 50 000, is a glycopeptide containing 13–16 % carbohydrate [3, 4].

The role of the glycopeptide in ATPase activity and cation transport has not been determined. Shamoo and Albers [9] has suggested that it may be an ionophore for Na^+. The glycopeptide may be involved in orienting the ATPase unit properly with regard to the plasma membrane [4]; also, it appears to contribute to the antigenicity of the ATPase [5]. The effect of antibody on ATPase activity varies from significant inhibition [5] to essentially no inhibition [10], depending upon the source of antigen and antibody. An antiserum raised against pig kidney outer-medulla ($Na^+ + K^+$)-ATPase inhibited ($Na^+ + K^+$)-ATPase in several other tissues. This antibody preparation also inhibited Na^+ efflux from human red blood cells, but only if exposed

to the inner-membrane surface [11]. Thus, the functions of the carbohydrates, and of the antigenic sites in the ATPase molecule, are not yet determined, and it is not known whether the glycopeptide component is even necessary for $(Na^+ + K^+)$-ATPase activity.

The binding specificity of plant lectins [12] has made them useful for investigating carbohydrates associated with macromolecules. They have been used to elucidate the molecular structures and functions of a variety of glycoproteins [13], including various surface glycoproteins on mammalian cells. The smaller polypeptide subunit, or glycopeptide, of $(Na^+ + K^+)$-ATPase contains glucosamine, galactosamine, sialic acid, and significant amounts of neutral sugar [4]. In this study, various lectins were used as probes in an attempt to elucidate possible roles for carbohydrate-containing regions of $(Na^+ + K^+)$-ATPase in enzyme activity and antigenicity.

METHODS

[^{3}H]Acetic anhydride was purchased from New England Nuclear (Boston, Mass.). Concanavalin A was purified from jack bean meal (Sigma Chemical Co., St. Louis, Mo.) by affinity chromatography [14]. An *N*-acetyl-D-glucosamine-specific lectin (M. S. Steinberg, personal communication) (*Dohlicos lablab*) was a gift from Dr M. S. Steinberg (Princeton University, Princeton, New Jersey). The crude protein was further purified by affinity chromatography on an ovomucoid column [15]. Wheat germ agglutinin was a gift from Dr M. M. Burger (Biozentrum, University of Basel, Switzerland). Soybean agglutinin was purchased from Miles Yeda Laboratories (Kankakee, Illinois). A fucose-specific lectin was purified by affinity chromatography from *Ulex europeus* seed meals [16] (S. E. Schumacher, Sandwich, Massachusetts). Phytohemagglutinin was purified from red kidney beans (*Phaseolus vulgaris*) (Jack Rabbit brand) as described earlier [16]. The lectins were checked for their agglutinating ability against 7-day-old chick embryo neural retina cells [17] or the appropriate type of human red blood cells. The lectins were acetylated with [^{3}H]acetic anhydride by a modification of the procedure described by Agrawal et al. [18] (Daniel, A., unpublished).

Lectins were dialyzed against 0.05 M Tris · HCl, pH 7.5, containing 1 mM EDTA for 1 day prior to use.

Enzyme preparation and assays

ATPase-containing microsomes were prepared from Electrophorus electric organ as previously described [19]. A solution containing 1.2 g Lubrol WX in 10 ml 0.1 M imidazole, pH 7.3, and 1 mM EDTA was added slowly at 0 °C to give a final Lubrol/protein ratio of 4 : 1. After 20 min, asolectin, 10 mg/ml, (Associates Concentrates, Woodside, N.Y.) was added to give equal weights of asolectin and microsomes. After centrifugation at $120\,000 \times g$ for 2 h, the supernatant, containing solubilized microsomes, was concentrated 4-fold using a stirred ultrafiltration cell under 70 lb/inch^2 N_2 (Amicon Corp., Lexington, Mass). The resulting solution was treated for 45 min with Bio-beads (Pharmacia, Uppsala, Sweden) to remove excess Lubrol. At least 50 % of the protein of this preparation can be recovered as the two major polypeptide components of $(Na^+ + K^+)$-ATPase by preparative polyacrylamide gel electrophoresis [5]. Protein was determined by the method of Lowry et al. [20]. Samples

were centrifuged briefly at 2000 rev./min before reading to remove precipitates.

ATP hydrolysis was determined by counting ^{32}P liberated from [^{32}P]ATP in a liquid scintillation counter as previously described [8]. The enzyme reactions proceeded for 10 min at 25 °C in a solution containing 0.05 M Tris base, pH 7.5, 1.5 mM $MgCl_2$, 5 mM dithiothreitol, 20 mM KCl, and 3 mM Tris/ATP; 62.5 mM NaCl was added to obtain $(Na^+ + K^+)$-ATPase activity.

The assay of potassium-nitrophenylphosphatase has also been described [21]. Reactions took place at 25 °C in 50 mM Tris · HCl, pH 7.5, with 10 mM tris-*p*-nitrophenylphosphate, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 20 mM KCl.

$(Na^+ + K^+)$-ATPase and potassium *p*-nitrophenylphosphatase assays were also run with 0.125 M α-methylglucoside in the incubation medium.

Immunodiffusion experiments

2 × 5 inch Ouchterlouny plates [22] were prepared using 10 ml 1 % Agar in 0.1 M veronal buffer, pH 8.4. 30-μl wells were used. Enzyme, lectins, and haptens were placed in the wells as described in Results and Discussion. Diffusion occurred at 4 °C in a humidified chamber. Rabbit antisera raised against lubrol-solubilized *Electrophorus electroplax* ATPase, prepared as in ref. 5, was kindly provided by Dr D. H. Jean.

Ouabain inhibition of $(Na^+ + K^+)$-ATPase

$(Na^+ + K^+)$-ATPase activity was measured continuously in the pH stat [23]. This method is advantageous for the study of ouabain inhibition as it can continuously measure the time course of $(Na^+ + K^+)$-ATPase over an extended period. Concanavalin A, α-methylglucoside and ouabain were added as shown in Results and Discussion (Fig. 4). Aminomethyl-1-propanol was used to neutralize H^+ liberated by ATP hydrolysis in the pH stat.

RESULTS AND DISCUSSION

Fig. 1 shows duplicate agar plates illustrating the effects of *Ulex* lectin and soybean lectin on the enzyme-antibody precipitin bands. In each case, neither the position nor the intensity of the two major bands is altered, save possibly for a slight inhibition of the outer band by *Ulex* lectin. Fig. 2 shows a similar experiment using concanavalin A and phytohemagglutinin A. The smaller inner band diminishes in size and essentially vanishes with increasing concanavalin A concentrations. Concanavalin A also alters the position of both bands. These effects are reversed by α-methylglucoside, a hapten inhibitor for concanavalin A.

Further experiments showed that concanavalin A does not bind to antiserum. If soy or *Ulex* lectin had shown an effect on enzyme-antibody precipitation, interpretation would have been difficult, as they do apparently bind to some component of the antiserum.

Thus, immunodiffusion experiments demonstrated combination of ATPase with concanavalin A, both by alteration of the migration rate of the resultant complex as reflected in the changed position of the remaining band, and by elimination of one precipitin band. Concanavalin A did not bind to antibody. Other lectins had little or no effect. This indicates that an α-glucoside or α-mannoside is probably present as

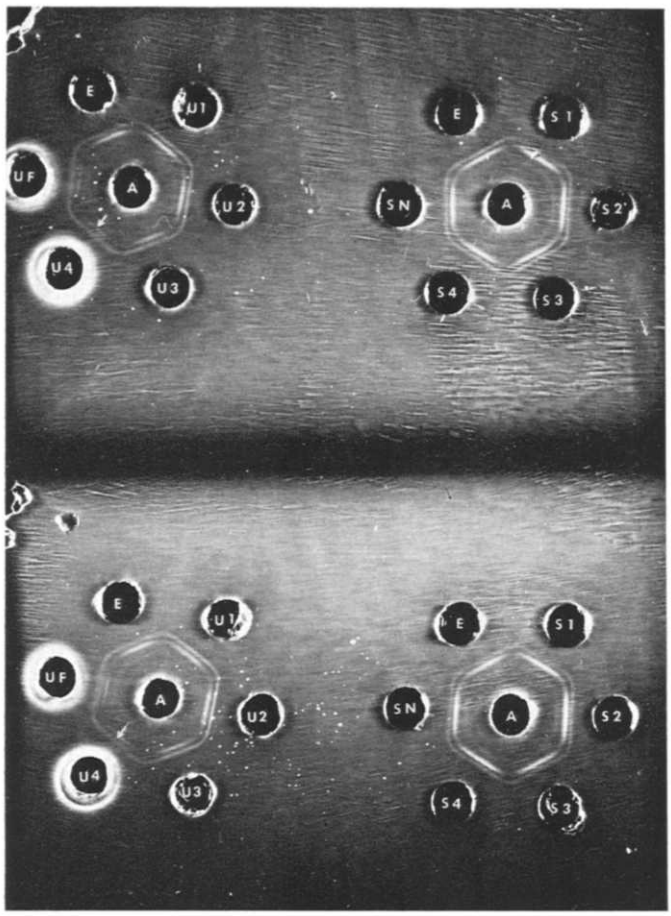

Fig. 1. Duplicate Ouchterlouny plates showing effects of *Ulex* and soybean lectins on ATPase antigen-antibody precipitation. Center wells (A) contain antiserum. Wells marked E contain enzyme only. Wells marked U1-U4 contain enzyme and *Ulex* lectin in approximate molar ratios of enzyme: *Ulex* = 4 : 1, 2 : 1, 1 : 1, 1 : 2. Well UF contains (1 : 2, molar ratio) enzyme: *Ulex* and 0.1 M fucose, a hapten for *Ulex* lectin which reverses it specific effects. The inner band seems slightly inhibited by *Ulex* lectin. Wells S1-S4 contain soybean lectin and enzyme in quantities similar to those of *Ulex* lectin and enzyme in wells U1-U4. Well SN also contains 0.1 M *N*-acetylgalactosamine, a hapten for soybean lectin. All outer wells of Figs 1 and 2 contain 225 μg enzyme.

the terminal or exposed carbohydrate in an antigenic region of the ATPase. (It was also possible to isolate a complex of concanavalin A · ATPase from a polyacrylamide gel column.)

Jean et al. [5] using preparations of antibody and antisera identical to those used here, demonstrated partial inhibition of $(Na^+ + K^+)$-ATPase by antisera which formed precipitin bands with solubilized enzyme. Control sera, which did not form precipitin bands with solubilized enzyme, did not alter enzyme activity.

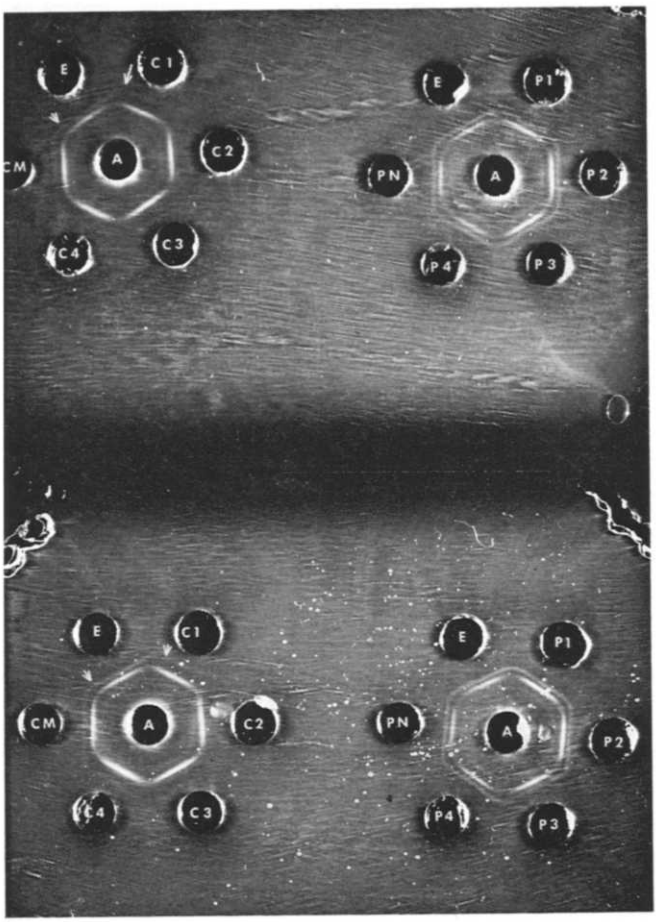

Fig. 2. Duplicate plates showing effects of concanavalin A and phytohemagglutinin. Wells are labeled in a manner similar to that of Fig. 1: C1-4 represents concanavalin A-enzyme, P1-4 represents phytohemagglutinin-enzyme. CM contains (2 : 1, molar ratio) concanavalin A-enzyme with 0.1 M α-methylglucoside, a hapten for concanavalin A; PN contains (2 : 1, molar ratio) phytohemagglutinin enzyme with 0.1 M *N*-acetylgalactosamine. Concanavalin A definitely shifts the position of the inner band and progressively eliminates the outer band (see arrows).

Effect of concanavalin A on enzyme activity

Fig. 3a shows the effect of progressively increasing amounts of concanavalin A on ATPase activity, in the presence and absence of α-methylglucoside. At moderate concentrations of concanavalin A, corresponding to molar ratios of 1–4 molecules of concanavalin A for one of the enzyme, there is inhibition of activity which is reversed by α-methylglucoside. At higher concentrations of concanavalin A, inhibition continues, but is not reversed by α-methylglucoside, even in large amounts. The molar ratios at which optimal reversible inhibition occurs correspond to those ratios which alter antigen-antibody precipitation (see Fig. 2).

Fig. 3B shows that concanavalin A apparently has only a non-specific effect on

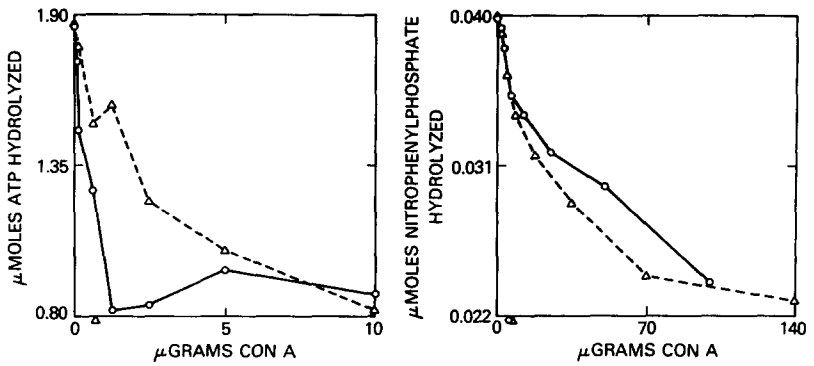

Fig. 3. (a) Effect of concanavalin A on $(Na^+ + K^+)$-ATPase activity. ○–○, with 2 μg enzyme, and the amount of concanavalin A shown on the *x*-axis; △- - -△, enzyme, concanavalin A, and 0.125 M α-methylglucoside, △ denotes the concanavalin A concentration giving a 1 : 1 molar ratio. The *y*-axis shows Na^+-dependent ATP hydrolysis. (b) Effect of concanavalin A on potassium *p*-nitrophenylphosphatase. *x*-axis shows μg concanavalin A; *y*-axis shows potassium *p*-nitrophenylphosphate-dependent hydrolysis of μmol/g protein per min. ○–○, without α-methylglucoside; △- - -△, with 0.125 M α-methylglucoside. Each reaction contained 25 μg enzyme. △ denotes the concentration of concanavalin A giving a 1 : 1 molar ratio.

the potassium *p*-nitrophenylphosphatase activity, since inhibition that does occur is not reversible by α-methylglucoside.

Concanavalin A thus has two effects on enzyme activity. There is an inhibition of the $(Na^+ + K^+)$-ATPase resulting from carbohydrate binding, and reversible by α-methylglucoside. There is no such effect on the potassium *p*-nitrophenylphosphatase. These effects, however, are superimposed on a non-specific irreversible inhibition of both nitrophenylphosphatase and ATPase, reflected in similar concanavalin A inhibition curves in the presence of α-methylglucoside. This non-specific adsorption has been observed in the binding of concanavalin A to myelin as well, accounting for up to 30 % of concanavalin A binding [24]. The concentration of concanavalin A causing specific, reversible $(Na^+ + K^+)$-ATPase inhibition was the same as that which reversibly inhibited precipitin band formation.

It is interesting that the maximum "specific" inhibition by concanavalin A is about 50 % of the total activity. This could be significant in relation to proposals for a 'half-of-sites' model of the enzyme [25]. The relationship of the antigenic regions of $(Na^+ + K^+)$-ATPase to its active sites has been uncertain. Recent studies have demonstrated an antibody which apparently binds to the larger polypeptide without inhibiting ATPase activity [10]. Other studies have shown varying degrees of inhibition by antibody preparations or antisera [5, 10, 26].

One of the precipitin bands observed here was not altered in intensity by concanavalin A, though its position in the gel was altered. The subunits of ATPase probably do not dissociate during immunodiffusion. Thus, this band probably represents an antigen-antibody reaction involving some membrane component other than ATPase.

While carbohydrate groups are generally associated with the outer cell membrane surface, concanavalin A could conceivably bind to the broken vesicles used in this study to sites corresponding to either the exterior or the interior of the intact cell.

ATPase, judging by its dimensions [4] and by its cation requirements [1], spans the plasma membrane. Jorgenson et al. [11] have shown that their antiserum to pig kidney enzyme, which inhibits $(Na^+ + K^+)$-ATPase in broken membrane preparations, also inhibits Na^+ efflux from erythrocytes if given access to the inner surface of the cell.

Effect of concanavalin A on ouabain inhibition of $(Na^+ + K^+)$-ATPase

In order to investigate the role of carbohydrate regions of the $(Na^+ + K^+)$-ATPase molecule on ouabain binding, the effect of concanavalin A on the rate of ouabain inhibition of ATPase was studied. Fig. 4 shows that addition of ouabain resulted in a decrease in rate of ATP hydrolysis; the rate of the decrease depended on concentrations of ouabain and K^+. Neither the rate nor the ouabain-K^+ interaction was altered by concanavalin A.

As ouabain binds to the outer surface of the cell, it is interesting that concanavalin A did not interfere perceptably with ouabain inhibition of ATPase. This is consistent with the lack of a specific effect on the potassium *p*-nitrophenylphosphatase. Thus K^+ sites are apparently not involved with the lectin-sensitive region of the ATPase.

The concanavalin-binding site of ATPase may thus be involved in Na^+ and/or ATP binding to the enzyme, or in regulation of a conformational change not necessary to the phosphatase reaction. Also, it could account for part of the antigenicity of ATPase in broken membrane preparations. The results reported here do not establish the relation of this site to subunits or to sidedness. Further experiments to elucidate these points are now in progress.

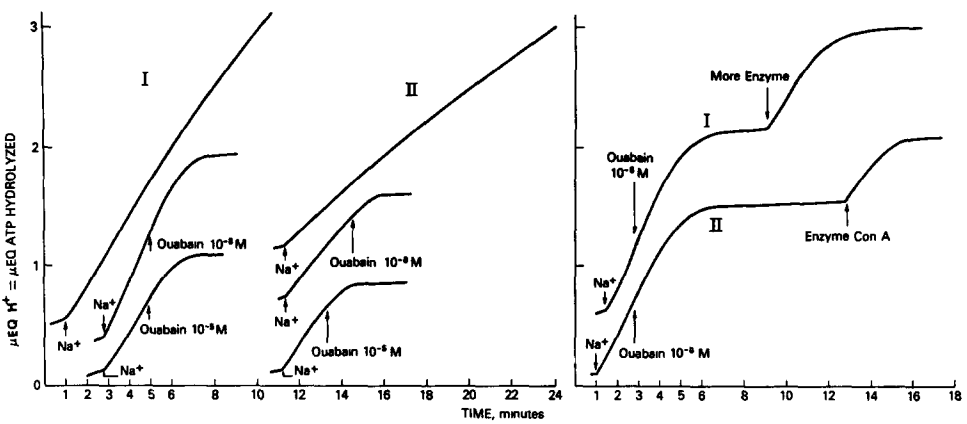

Fig. 4. (a) (I) Ouabain inhibition of $(Na^+ + K^+)$-ATPase, with and without concanavalin A (1 : 3 molar ratio of enzyme : concanavalin A), 1 mM KCl. X-axis is time in minutes; Y axis is μequivalents H^+ neutralized by pH stat. To start the $(Na^+ + K^+)$-ATPase reaction, NaCl was added to give final $[Na^+] = 62.5$ mM. Ouabain was added at the time shown by the arrow. The top curve is a control experiment in which ouabain was never added. (II) Conditions were similar to I except that enzyme had been combined with concanavalin A (1 : 3, molar ratio) 30 min before reaction. Twice as much enzyme protein was used as in I. (b) Conditions and labeling similar to Fig. 3a, except that [KCl] was 30 mM. Curve I, enzyme only; curve II, enzyme-concanavalin A, 1 : 3, molar ratio.

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