Direct Stimulation of Na^+-K^+-ATPase and Its Glucosylated Derivative By Aldose Reductase Inhibitor

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SUMMARY

In the presence of 10^{-8} M concentrations of the aldose reductase inhibitor AL 1576, there is a 20–30% increase in the rate of hydrolysis of near-saturating concentrations of ATP by bovine renal Na^+-K^+-ATPase. When bovine renal Na^+-K^+-ATPase is reacted with glucose 6-phosphate in the presence of 10^{-8} M concentrations of AL 1576 or 10^{-6} M concentrations of a second aldose reductase inhibitor, sorbinil, glucosylation occurs. Whereas sorbinil has no effect on ATP hydrolysis by the glucosylated Na^+-K^+-ATPase, 10^{-8} M AL 1576 causes a shift in the kinetics of hydrolysis of ATP from substrate inhibition to normal substrate activation. The aldose reductase inhibitors interact with the enzyme at the low-affinity ATP-binding site. *Diabetes* 36:716–20, 1987

Decreases in the rate of hydrolysis of near-saturating concentrations of ATP by the Na^+-K^+-ATPase of the sciatic nerve (1), dorsal root ganglia (2), and glomeruli (3) are observed in streptozocin (STZ)-induced diabetes in rats. Inhibition of K^+ transport as well as inhibition of ATP hydrolysis by the Na^+-K^+-ATPase of the lens epithelium of galactosemic rats has been reported (4). In human diabetes, the Na^+-K^+-ATPase isolated from either the renal medulla (5) or lens epithelium (6) displays inhibited ATP hydrolysis at near-saturating concentrations of ATP. Apparent substrate inhibition kinetics of ATP hydrolysis are observed (5,6). Na^+-K^+-ATPase from nondiabetic individuals displays either substrate activation or Michaelis-Menten ATP hydrolysis kinetics (5,6). Glucosylation of the pure bovine renal Na^+-K^+-ATPase with glucose 6-phosphate followed by $NaBH_4$ reduction yields an enzyme with reduced ATP hydrolysis at near-saturating ATP concentrations and substrate-inhibition kinetics (5). Treatment of bovine lenses in culture with high glucose concentrations leads to inhibition of Na^+-K^+-ATPase–dependent K^+ influx as well as a shift to substrate-inhibition ATP hydrolysis kinetics (6). Sorbinil in vivo appears to restore pump function in the peripheral nerve, renal glomerulus, and arterial smooth muscle of alloxan- or STZ-induced diabetes (1–3,7). Because sorbinil restores tissue *myo*-inositol levels in these model systems, it has been proposed that the increased concentration of membrane phosphoinositides are responsible for the enhancement of Na^+-K^+-ATPase activity (7,8). Alternatively, it has been proposed that phosphoinositide breakdown releases inositol 1,4,5-trisphosphate and diacylglycerol, activating protein kinase indirectly, which in turn phosphorylates Na^+-K^+-ATPase, thus inhibiting it (9). Sorbinil in this scheme prevents this phosphorylation by maintenance of normal phosphoinositide levels.

Neither of these proposed schemes addresses the fact that Na^+-K^+-ATPase may be inhibited due to the covalent attachment of glucose to critical amino groups on the enzyme. These amino groups are known to play an important role for both Na^+ and K^+ interaction with the enzyme (11,12). Therefore, sorbinil, if it is to restore normal pump-dependent K^+ influx or Na^+ efflux, may *1*) force a defective pump to function by direct binding or release of a cellular modulator or *2*) stimulate the synthesis of more Na^+-K^+-ATPase molecules.

Our experiments were designed to determine if sorbinil and a second aldose reductase inhibitor (AL 1576) have any effect on pure renal Na^+-K^+-ATPase and its glucosylated analogue.

MATERIALS AND METHODS

Bovine kidneys were provided by the Great American Veal Company within 2 h of death. Aldose reductase inhibitor

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TABLE 1
Effects of sorbinil and AL 1576 on hydrolysis of ATP by bovine renal Na^+-K^+-ATPase

Sorbinil		AL 1576	
Concn (M)	PO_4 release ($\mu M \cdot mg^{-1} \cdot min^{-1}$)	Concn (M)	PO_4 release ($\mu M \cdot mg^{-1} \cdot min^{-1}$)
1×10^{-3}	22.3 ± 1.3*	2×10^{-6}	22.5 ± 0.2*
2×10^{-4}	25.0 ± 1.3	1×10^{-7}	25.8 ± 0.5
2×10^{-5}	26.4 ± 0.2	2×10^{-7}	26.8 ± 0.2*
1×10^{-6}	26.1 ± 0.3	1×10^{-8}	27.1 ± 0.1*
1×10^{-7}	25.8 ± 0.5	2×10^{-8}	30.4 ± 0.2*
2×10^{-8}	26.9 ± 0.2	1×10^{-9}	29.1 ± 0.2*
2×10^{-9}	27.3 ± 0.9	2×10^{-9}	28.2 ± 0.2*
1×10^{-10}	26.4 ± 0.5	1×10^{-10}	27.1 ± 0.1
0	26.4 ± 0.2	0	26.2 ± 0.2

All experiments were performed 4 times.
*Experiments in which, by 2-tailed *t* test, hydrolysis rate is significantly different from rate in absence of any drug.

AL 1576 was provided by Alcon (Fort Worth, TX), sorbinil was provided by Pfizer (Groton, CT). ATP (disodium salt) and glucose 6-phosphate (sodium salt) were obtained from Boehringer Mannheim (Indianapolis, IN), FITC was from Sigma (St. Louis, MO), and [^{3}H]ouabain (1 μCi/μl) and D-[1-^{14}C]glucose 6-phosphate (52 mCi/mM) were from New England Nuclear (Boston, MA).

Na^+-K^+-ATPase (0.5 mg/ml), free of ouabain-insensitive ATPase activity, was isolated from the bovine renal medulla as described previously (sp act at 5 mM [Mg^{2+}-ATP] = 24 $\mu M\ P_i \cdot mg^{-1} \cdot min^{-1}$) (12). It was dialyzed for 24 h against 30 mM imidazole buffer, pH 7.4, containing 150 mM KCl. The glucosylation of the renal Na^+-K^+-ATPase was achieved by the addition of glucose 6-phosphate (final concn 16.7 mM) to a solution containing the dialyzed enzyme and 6.2 mM ATP. In selected experiments, AL 1576 (final concn 10^{-8} M) or sorbinil (final concn 10^{-6} M) was added before the addition of the cold or radiolabeled glucose 6-phosphate. The pH of the reaction solution was adjusted to and maintained at 6.7. After 90 min, the reaction mixture was diluted 10-fold with cold 1 M histidine, pH 7.0, containing 50 mM sodium cyanoborohydride (Aldrich, Milwaukee, WI). The cyanoborohydride reduction proceeded at 4°C for 60 min. The Na^+-K^+-ATPase was isolated from this mixture by centrifugation at 100,000 × *g* for 1 h. The pelleted enzyme was rinsed twice by resuspension in 1 mM imidazole EDTA, pH 7.4, and recentrifuged for 1 h at 100,000 × *g*. Control samples were prepared with an identical procedure except for elimination of glucose 6-phosphate. Sodium cyanoborohydride reduction had no adverse effect on Na^+-K^+-ATPase.

The hydrolysis of ATP in the absence and presence of the aldose reductase inhibitors by the renal Na^+-K^+-ATPase preparations was performed at 37°C in 30 mM imidazole buffer, pH 7.4, containing 130 mM NaCl, 20 mM KCl, and varying concentrations of $MgCl_2$ and ATP (5,6,13). In all cases, <5% of the ATP was hydrolyzed to ensure measurement of the initial rate. There was no detectable ouabain-insensitive ATP hydrolysis.

For experiments to determine the kinetics of hydrolysis of ATP, the data were fitted to the algebraic rearrangement of the second-degree rational rate function

$$v = \frac{V_{max}}{1 + K/[ATP] + D/(A + [ATP])} \quad (1)$$

where V_{max} is the velocity at infinite substrate concentration, and *K*, *D*, and *A* are constants having dimensions of concentration (14).

All curves fit to the experimental points were obtained, without being statistically weighted, with either the Prophet (Bolt, Baranek, and Newman, Cambridge, MA) computer procedure FITFUN or a fitting routine developed by W. H. Garner for a Tektronix 4170 computer with the Marquardt algorithm. Both programs fit the data by minimizing the sum of the squares. In all cases, $P < .001$, and r^2 is reported for all individual data sets. A near-normal distribution of the residuals at ~0 was obtained for most of the fits presented here, which indicates that Eq. 1 is adequate in describing the data.

Measurement of ouabain binding. Na^+-K^+-ATPase (1 mg/ml) was dialyzed against 30 mM imidazole buffer, pH 7.0, containing 150 mM NaCl and 1 mM EDTA. All binding studies were performed in polycarbonate tubes designed for the Beckman airfuge. Twenty-seven picomoles of Na^+-K^+-

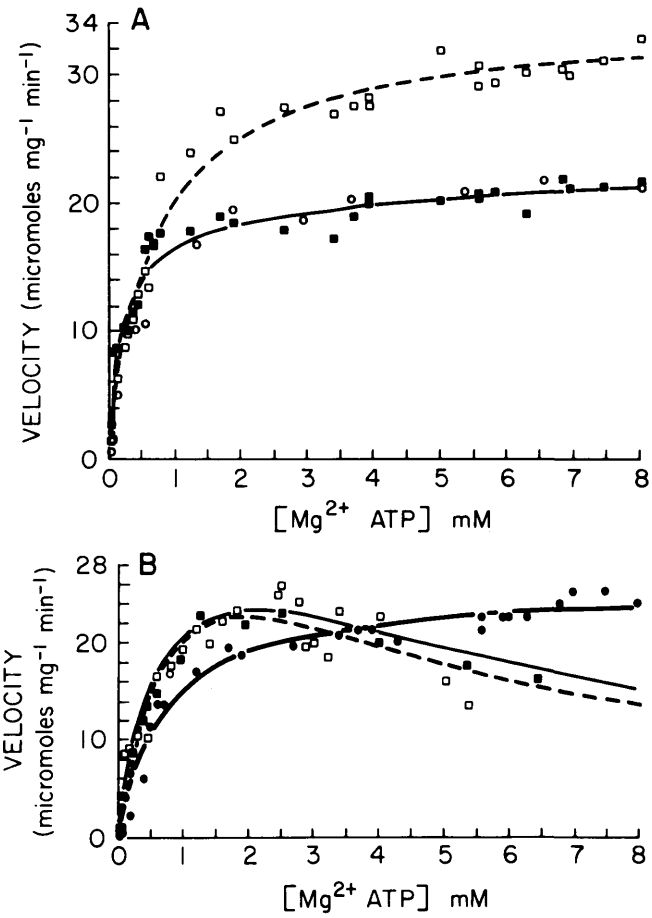

FIG. 1. *A*: plots to evaluate hydrolysis of ATP by native Na^+-K^+-ATPase (■) in presence of sorbinil (○) and in presence of AL 1576 (□). *B*: plots to evaluate hydrolysis of ATP by glucosylated Na^+-K^+-ATPase (■) in presence of sorbinil (□) and in presence of AL 1576 (●).

TABLE 2
Computed kinetics values for the hydrolysis of ATP

	V_{max} (μpmol · mg^{-1} protein · min^{-1})	*K* (mM)	*D* (mM)	*A* (mM)	*K* + *D* (mM)	R^2
Unmodified Na^+-K^+-ATPase						
Control	30 ± 1	0.3 ± 0.03	9.7 ± 4	18 ± 5	10.0	.996
+AL 1576	37 ± 1	0.7 ± 0.3	4 ± 1	37 ± 1	4.7	.996
+Sorbinil	30 ± 3	0.9 ± 0.5	5 ± 2	15 ± 4	5.9	.993
Glucosylated Na^+-K^+-ATPase						
Glucosylated	0.1 ± 0.1	0.03 ± 0.02	−42 ± 10	−52 ± 20	−42	.994
+Sorbinil	0.2 ± 0.1	0.1 ± 0.1	−40 ± 20	−47 ± 17	−40	.994
+AL 1576	27 ± 2	0.7 ± 0.1	0.9 ± 1	20 ± 8	1.6	.994

V_{max}, velocity at infinite substrate concentrations; *K*, *D*, and *A*, constants having dimensions of concentration. Multiple R^2 values represent goodness of fit.

ATPase were placed in polycarbonate tubes in the presence and absence of specified concentrations of sorbinil or AL 1576. After a 1-h preincubation period, 0.5 μCi of [^{3}H]ouabain (20 Ci/mmol) was added, and the tubes were incubated for 3 h at room temperature. Sodium dodecyl sulfate (SDS)–denatured Na^+-K^+-ATPase was used as the blank for each experiment. After incubation, the samples were centrifuged at 105,000 × *g* for 30 min with the Beckman airfuge. Trichloroacetic acid (20%) was used to precipitate the SDS-denatured ATPase in the blanks before centrifugation. The supernatant was removed, and an aliquot was counted. The pellets were rinsed with deionized water, dissolved in 2% SDS, and counted.

Fluorescein isothiocyanate modification. Na^+-K^+-ATPase was suspended to a concentration of 1 mg/ml in 50 mM Tris buffer, pH 9.0, containing 100 mM NaCl and 5 mM EDTA and various concentrations of sorbinil, AL 1576, or ATP (15). FITC was added to a concentration of 1 × 10^{-5} M. The reaction was allowed to proceed at room temperature in the dark for 2 h. The unreacted FITC was removed by centrifugation at 105,000 × *g* for 30 min. The resultant pellet was rinsed with 1 mM imidazole EDTA buffer, pH 7.4, and suspended in the imidazole EDTA buffer containing 100 mM sucrose.

Separation of α- and β-subunits. The α- and β-subunits of Na^+-K^+-ATPase were separated by fast protein liquid chromatography (FPLC) with a Superose 6 column. The eluent was 0.050 M NaH_2PO_4, pH 6.0, containing 0.1% SDS. The Na^+-K^+-ATPase was denatured and deaggregated in 0.050 M phosphate buffer containing 1% SDS and 0.1% β-mercaptoethanol before loading. A typical run took 2 h at a flow rate of 0.2 ml/min. The elution was monitored at 280 nm. When FITC-labeled Na^+-K^+-ATPase was used, a second detection at 495 nm was also used. SDS–polyacrylamide gel electrophoresis was used to characterize the molecular-weight distribution of the eluted fractions. In experiments in which a radiolabeled enzyme was used, 0.4-ml samples were collected, suspended in 20 ml of Liquiscint (National Diagnostics), and counted.

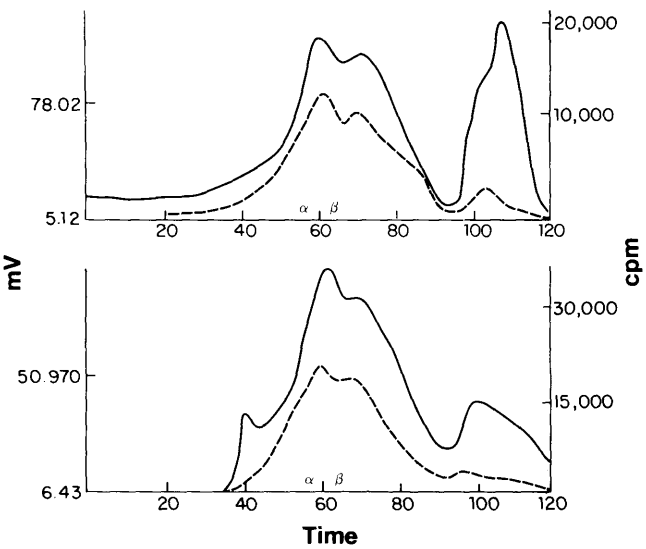

FIG. 2. Elution profiles for separation of α- and β-subunits of Na^+-K^+-ATPase by fast protein liquid chromatography on Superose 6. Na^+-K^+-ATPase of 0.5 mg was loaded in each case. *Top*: chromatography of Na^+-K^+-ATPase glucosylated in presence of sorbinil (10^{-5} M). *Bottom*: chromatography of Na^+-K^+-ATPase glucosylated in presence of AL 1576 (10^{-6} M). *Solid line* is absorbance at 280 mM recorded as mV. *Dashed line*, radioactivity measured on 0.4-ml fractions of eluent. Flow rate was 0.2 ml/min.

RESULTS

Table 1 lists the results of experiments designed to study the effects of sorbinil (*S*-6-fluorospirochroman-4,4′-imidazolidine-2′,5′-dione) and AL 1576 (*S*-2,7-difluorofluorene-9,4′-imidazolidine-2′,5′-dione) on the Na^+-K^+–dependent hydrolysis of 3 mM Mg^{2+}-ATP by bovine renal Na^+-K^+-ATPase. There is a significant increase in the rate of hydrolysis of ATP at AL 1576 concentrations between 10^{-9} and 10^{-7} M. Higher concentrations of AL 1576 appear to be inhibitory. Except at relatively high concentrations, sorbinil has no effect on ATP hydrolysis by bovine renal Na^+-K^+-ATPase.

To verify the observation that AL 1576 stimulates ATP hydrolysis by Na^+-K^+-ATPase, the kinetics of hydrolysis of ATP

TABLE 3
Ouabain binding to Na^+-K^+-ATPase: effects of aldose reductase inhibitors

	Ouabain binding (mol/mol αβ-dimer)
Sorbinil (M)	
1.2×10^{-5}	0.97 ± 0.16 (4)
1.2×10^{-6}	1.18 ± 0.26 (2)
AL 1576 (M)	
2.4×10^{-6}	1.06 ± 0.08
2.4×10^{-7}	0.98 ± 0.06

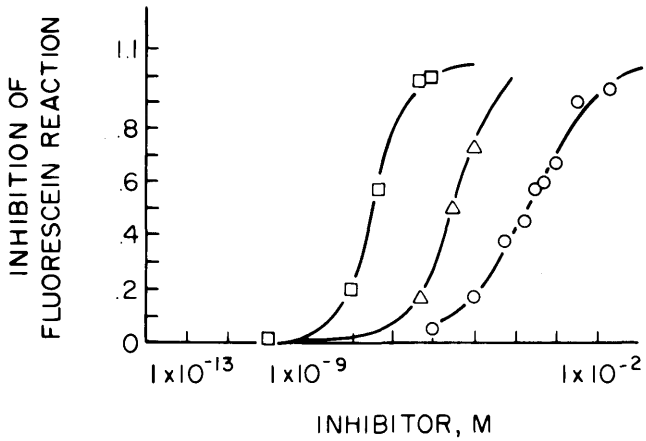

FIG. 3. Dose-response curves for inhibition of FITC modification of Na^+-K^+-ATPase by ATP (○), sorbinil (△), and AL 1576 (□).

were determined in the presence of 10^{-8} M AL 1576, in the presence of 10^{-6} M sorbinil, and in the absence of both drugs (Fig. 1*A*). The curves through the experimental points in Fig. 1*A* were obtained with Eq. 1; the resulting constants are listed in Table 2. There is a significant increase in V_{max} when AL 1576 is present in the experimental solutions. Sorbinil has no effect on the kinetics of hydrolysis of ATP by Na^+-K^+-ATPase.

Renal Na^+-K^+-ATPase was then modified with glucose 6-phosphate in the presence of 10^{-6} M AL 1576, in the presence of 10^{-5} M sorbinil, and in the absence of both drugs. In all cases, Na^+-K^+-ATPase was glucosylated (2–4 mol glucose 6-phosphate/mol Na^+-K^+-ATPase were incorporated). Separation of the α- and β-subunits of the Na^+-K^+-ATPase, glucosylated in the presence of sorbinil and in the presence of AL 1576, was accomplished as described in MATERIALS AND METHODS. The elution profiles shown in Fig. 2 for sorbinil (*top*) and for AL 1576 (*bottom*) indicate that the label is equally distributed between the α- and β-subunits. This result is similar to that reported previously for the enzyme glucosylated in the absence of both drugs (5).

Therefore, if either aldose reductase inhibitor is to have a direct effect on Na^+-K^+-ATPase function in diabetes, it must change the function of the glucosylated derivative. To test this hypothesis, the kinetics of hydrolysis of ATP by the glucosylated enzyme were determined in the presence of 10^{-8} M AL 1576 and in the presence of 10^{-6} M sorbinil. The results are graphed in Fig. 1*B* for glucosylated ATPase and for the same enzyme preparation in the presence of AL 1576 and sorbinil. The curves through the experimental points were obtained with Eq. 1. The kinetic parameters from these fits are listed in Table 2. The parameters for the glucosylated Na^+-K^+-ATPase determined in the presence of sorbinil are similar to those of the glucosylated Na^+-K^+-ATPase alone (D and $[K + D] < 0$, indicative of substrate-inhibition kinetics). The kinetics parameters for the glycosylated enzyme determined in the presence of AL 1576 are similar to the unmodified control enzyme (D and $[K + D] > 0$, indicative of substrate-activation kinetics). Because of the dramatic effect that AL 1576 has on ATP hydrolysis by both Na^+-K^+-ATPase and its glucosylated derivative, experiments were initiated to locate the site of interaction of the aldose reductase inhibitor with Na^+-K^+-ATPase.

Cardiotonic steroids, e.g., ouabain, bind to a specific inhibitory site on the α-subunit of Na^+-K^+-ATPase (16). To determine if this was also the site of action of the aldose reductase inhibitors, the binding of radiolabeled ouabain in the presence and absence of sorbinil and AL 1576 was measured. The results indicate that ouabain binds to bovine Na^+-K^+-ATPase at a ratio of 1 mol/mol αβ-dimer and that ouabain binding is unaffected by either sorbinil or AL 1576 (Table 3).

Na^+-K^+-ATPase apparently has two binding sites for ATP, one with high affinity (K_{50} in μM range) and one site with low affinity (K_{50} in mM range). From the kinetics experiments, ATP hydrolysis at near-saturating substrate concentrations is altered by AL 1576, thus implicating drug interaction with the low-affinity ATP-binding site. FITC modifies one lysine residue in the low-affinity ATP-binding site (15,18–20), and FITC modification totally inhibits Na^+-K^+-ATPase activity (ATP hydrolysis at near-saturating concentrations of Mg^{2+}-ATP in presence of 130 mM Na^+ and 20 mM K^+). Therefore, the effects of the two aldose reductase inhibitors on FITC modification was used to see if the low-affinity ATP-binding site is the site of aldose reductase inhibitor interaction with the pump. FITC modification in the presence and absence of the aldose reductase inhibitors was measured in two ways: by the loss of Na^+-K^+-ATPase activity and by FPLC. In the absence of ATP or the aldose reductase inhibitors, a 2-h reaction of bovine Na^+-K^+-ATPase with FITC at alkaline pH totally inhibits Na^+-K^+-ATPase activity (15). ATP, sorbinil, and AL 1576 interfere with FITC inhibition as shown in Fig. 3. The K_{50} values for inhibition of the FITC modification of Na^+-K^+-ATPase (measured by inhibition of Na^+-K^+-ATPase activity) are 2.9×10^{-3} M, 3.9×10^{-6} M, and 2.0×10^{-8} M for ATP, sorbinil, and AL 1576, respectively. Figure 4 shows representative elution profiles for the separation of the α- and β-

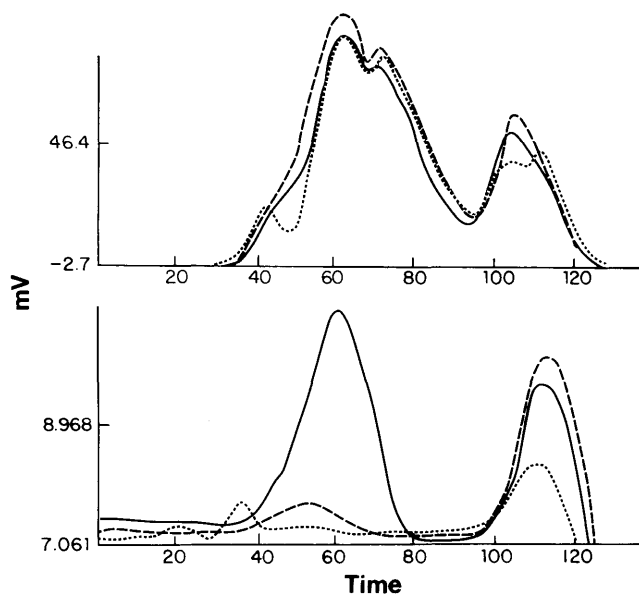

FIG. 4. Elution profiles for fast protein liquid chromatography separation of α- and β-subunits of Na^+-K^+-ATPase after FITC modification in presence of sorbinil (*dashed line*), AL 1576 (*dotted line*), and in absence of drugs (*solid line*). Elution profiles in *top panel* were obtained at 280 mM; elution profiles in *bottom panel* were obtained at 495 mM.

subunits of Na^+-K^+-ATPase modified with FITC in the presence of 10^{-6} M AL 1576, in the presence of 10^{-5} M sorbinil, and in the absence of both drugs. The elution was monitored at two wavelengths: 280 nM (Fig. 4, *top*) to follow the subunit separation and 495 nM (Fig. 4, *bottom*) to follow the fluorescein moiety. FITC modifies only the α-subunit of bovine Na^+-K^+-ATPase, and this modification is inhibited by both aldose reductase inhibitors.

DISCUSSION

The aldose reductase inhibitors sorbinil and AL 1576 appear to interact with Na^+-K^+-ATPase directly. They compete with FITC for the low-affinity ATP-binding site on the enzyme. AL 1576 stimulates pump-dependent ATP hydrolysis by 15–20%. Sorbinil, on the other hand, has little or no effect on ATP hydrolysis by bovine renal Na^+-K^+-ATPase. The chroman ring of sorbinil and the fluorene moiety of AL 1576 are the portions of the two inhibitors most likely responsible for the inhibition of aldose reductase. These groups are probably also responsible for the binding of these drugs to the low-affinity ATP-binding site of Na^+-K^+-ATPase. This site has a higher apparent affinity for the fluorene group than for the chroman moiety, which may explain the difference in effect of the drugs on ATP hydrolysis.

From the data presented in Fig. 2, it appears that neither aldose reductase inhibitor is capable of preventing the in vitro glucosylation of the pump. The amines most likely glucosylated at neutral pH are the Bohr amines described previously by Skou and Esmann (10,11). These amines appear to be involved more with cation binding than with substrate interaction with the enzyme (11). The reactive amine in the low-affinity site has a more normal p*K* value (9.0–9.5) and is relatively unreactive at neutral pH to amine-modifying agents that react specifically with the deprotonated form of an amino group. Whether either inhibitor is capable of preventing the in vivo glucosylation of the Na^+ pump in the presence of elevated glucose concentrations remains to be elucidated. Both would be expected to protect the amine in the adenine-binding site.

From the data presented here, AL 1576 binding to the glucosylated Na^+-K^+-ATPase shifts the kinetics of ATP hydrolysis from substrate inhibition to substrate activation. The result indicates that AL 1576 may be capable of forcing the defective (glucosylated) pump to function normally. Although it does interact with Na^+-K^+-ATPase in a manner similar to AL 1576, sorbinil appears to be incapable of modifying the kinetics of hydrolysis of ATP by the glucosylated pump. Further experiments are necessary to determine if this difference between the aldose reductase inhibitors is also reflected in pump-dependent Na^+ and K^+ transport.

Unlike AL 1576, sorbinil does not directly affect ATP hydrolysis by the normal or diabetic Na^+-K^+-ATPase. However, there are ample data that suggest sorbinil indirectly restores in vivo pump function (1–3). Sorbinil not only inhibits sorbitol accumulation but also restores tissue *myo*-inositol levels. *myo*-Inositol (7) and protein kinase C (9) agonists also restore Na^+-K^+-ATPase–dependent ATP hydrolysis. These observations have led to the suggestion of protein kinase C involvement in Na^+-pump inhibition in diabetes. However, there is no direct evidence of protein kinase phosphorylation of the Na^+-K^+-ATPase in diabetic tissue. ATP, K^+, and Na^+ interaction with protein kinase–phosphorylated Na^+-K^+-ATPase also remains to be elucidated.

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