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$(Na^+ + K^+)$-STIMULATED ATPASE OF HUMAN KIDNEY, NORMAL AND ADENOCARCINOMA. PHOSPHORYLATION AND INHIBITION BY ANTITUMOR PROTEINS.

Asgar Zaheer[†], Diwan Singh[≠] and Rex Montgomery[†]
Departments of Biochemistry[†] and Anatomy[≠]
University of Iowa, Iowa City, Iowa 52242

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SUMMARY: $(Na^+ + K^+)$-ATPase was purified from human kidney of normal and tumor tissue with specific activities of 100.0 and 16.6 μmol Pi/mg/h, respectively. The antitumor proteins, macromomycin, largomycin, and NSC 327459 (50 μg/ml each) caused 70 to 90% inhibition of $(Na^+ + K^+)$-ATPase from tumor tissue, whereas auromomycin had no effect. $(Na^+ + K^+)$-ATPase from both sources could be phosphorylated by rabbit muscle protein kinase; there was 3 to 6-fold stimulation of phosphorylation by cyclic AMP. Phosphorylation resulted in 70 to 80% decrease in $(Na^+ + K^+)$-ATPase activity, and caused the normal enzyme to become sensitive to inhibition by macromomycin.

Earlier studies (1,2) showed that some $(Na^+ + K^+)$-stimulated ATPases were inhibited by the antitumor protein, macromomycin, isolated from Streptomyces macromomyceticus. It was of particular interest to note that the enzyme from human adenocarcinoma was inhibited whereas that from normal human kidney tissue was not. This difference has been noted in all cases studied, the present paper examining the two types of $(Na^+ + K^+)$-ATPase from a single human kidney with a large tumor that was well circumscribed.

In the course of examining the effects of antitumor proteins on the metabolism of tumor cells in tissue culture, the possibility was considered that the regulation of protein kinase was involved. The present studies demonstrate phosphorylation of human kidney $(Na^+ + K^+)$-stimulated ATPase by a protein kinase resulting in reduced specific activity of the enzymes; the phosphorylated enzymes are more susceptible to inhibition by macromomycin.

Abbreviations used: $(Na^+ + K^+)$-ATPase-ATP phosphohydrolase, EC 3.6.1.3; SDS-sodium dodecyl sulfate; NaI-sodium iodide; EDTA-(ethylenedinitrilo)-tetra acetic acid disodium salt.

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EXPERIMENTAL PROCEDURES

Materials - Protein kinase from rabbit skeletal muscle was purified as described by Beavo et.al. (3). Antitumor proteins, macromomycin and auromomycin, were prepared from Streptomyces macromomyceticus as described earlier (4,5). Purification of the antitumor protein largomycin from culture filtrate of Streptomyces pluricolorescens will be described elsewhere.[1] Antitumor protein-NSC 327459 was obtained from Dr. J. C. Cradock, National Cancer Institute. [γ-^{32}P] ATP was purchased from New England Nuclear. ATP and ouabain were obtained from Sigma.

Purification of $(Na^+ + K^+)$-ATPase - Human kidney, normal and tumor tissue was obtained. The adenocarcinoma was a clear cell type, well circumscribed and occupying one pole of the kidney. The tumor tissue was dissected from normal tissue that showed no pathology. The tissues were frozen at -20°C for a short time before being used for the enzyme preparations. The $(Na^+ + K^+)$-ATPase was purified essentially as described by Braughler and Corder (6) for human renal tissue with several modifications as follows. All operations were carried out at 0-4°C unless otherwise specified. Frozen kidney tissues were thawed in 100mM Tris (pH 7.0) containing 0.25 M sucrose. Extraneous fat, large vessels, and the capsule were removed. Each of the tissues was homogenized in 10 volumes of Tris-sucrose solution in a Waring Blender at high speed for 40 sec. and centrifuged at 10,000 x g for 20 min. The pellet was rehomogenized in 5 volumes of Tris-sucrose and centrifuged as before. The 10,000 x g supernatant was further contrifuged at 105,000 x g for 1 hour (Beckman L5-50, rotor Ti 50.2) and the pellet was suspended in 25mM imidazole buffer (pH 7.0) containing 1mM disodium EDTA at a protein concentration of approximately 5 mg/ml.

The removal of ouabain insensitive ATPase activity present in the membrane preparations was carried out by treatment with 2M NaI (7). A solution containing 12g NaI in 26 ml of distilled water, 2 ml of 0.002 M EDTA (pH 7.1), 4 ml 0.1M dithiothreitol and 2 ml of 0.8M Tris-HCl buffer (pH 8.0) was added to 10 ml of membrane preparation and the suspension was stirred gently for 30 min. in an ice-bath. To this was added 1mM EDTA (100ml) with thorough mixing, which reduced the concentration of NaI to 0.8M. The suspension was then centrifuged for 60 min. at 77,000 x g and the residue so obtained was resuspended in 10 ml of 0.1M sucrose solution containing 1mM EDTA (pH 7.1) and diluted to 100 ml. After centrifugation at 160,000 x g for 15 min., the precipitate was washed twice, first with 1mM EDTA containing sucrose and then with EDTA (pH 7.1) in the absence of sucrose. The pellet was recovered each time by centrifuging at 160,000 x g and finally suspended in 1mM EDTA (pH 7.1).

The NaI-treated enzyme was solubilized with sodium dodecylsulfate as described earlier (1). The NaI-treated enzyme (protein concentration, 1.5 mg/ml) was treated for 30 min. at 20°C with 2mM EDTA, 50mM imidazole, pH 7.5, the incubation being started by the addition of a freshly prepared solution of SDS (2 mg/ml) to a final concentration of 0.6 mg/ml. An aliquot of the resulting suspension was layered on a discontinous density gradient of three successive layers of sucrose: 29.4%, 15%, and 10% (w/v) sucrose in 25mM imidazole, 1mM EDTA, pH 7.5, and was centrifuged at 45,000 rpm for 135 min. (Beckman L5-50, Ti 50.2 rotor). The pellet was collected and suspended in 1mM EDTA, pH 7.0.

Phosphorylation Assay - The phosphorylation reaction mixture, contained in a total volume of 100 µl, 50 mM sodium acetate, pH 6.2, 5mM theophylline, 5 µM cyclic AMP (when added), 5mM $MgCl_2$, 100 µM [γ^{32}P]ATP (50-70 cpm/pmol), 6 µg of rabbit-skeletal muscle protein kinase and 100 µg of $(Na^+ + K^+)$-ATPase protein. The reaction was initiated by adding the enzyme protein kinase, continued at 37°C for 5 min. and was terminated by the addition of 2.0 ml of ice-cold 20% trichloroacetic acid. The enzymes were also incubated under identical conditions in absence of ATP and Mg^{2+}. The contents of tubes

1. Vandré, D.D. and Montgomery, R., manuscript in preparation.

were mixed well and the protein allowed to precipitate for 15 min. The acid-insoluble protein was collected on glass fiber filter disks (Whatman GF/A), which had been washed and pre-equilibrated with 20% trichloroacetic acid. After the precipitated proteins had been thoroughly washed with ice-cold 5% trichloroacetic acid (about 15 ml per disk), the filters were dried and counted for radioactivity in 5 ml of toluene-based scintillation fluid containing 0.375% (w/v) 2,5- diphenyloxozole, and 0.01% (w/v) 2,2´-p-phenylene-bis-(4-methyl-5-phenyloxazole).

Preliminary results indicated that under these experimental conditions the phosphorylation of $(Na^+ + K^+)$-ATPase in 5 min. was 70-75% of maximum.

$(Na^+ + K^+)$-ATPase Assay - $(Na^+ + K^+)$-ATPase activity was determined as described earlier (8). For assay of the phosphorylated enzyme, the phosphorylation reaction was continued for 5 min. after which time the mixture was cooled quickly to 0°C and the enzyme suspension was quickly washed twice (centrifugation time not more than 1-2 min.) in 10 mM Tris-HCl buffer, pH 7.0. The enzyme was preincubated with ouabain and macromomycin for 5 min. at 37°C in the assay medium containing no ATP. Following the preincubation, the reaction was started by the addition of 3mM ATP.

Phosphate Estimation - Inorganic phosphate was assayed by the method of Fiske and SubbaRow (9).

Protein Determination - Protein was estimated by the method of Lowry et.al. (10).

RESULTS AND DISCUSSION:

The enzyme $(Na^+ + K^+)$-stimulated ATPase has been purified from a variety of normal and tumor tissues. Enzyme preparations from tumor cells appear to be considerably less active (1,11,12). Barclay and Terebus-Kekish (13) have reported that plasma membrane preparations enriched in $(Na^+ + K^+)$-ATPase isolated from Morris hepatoma 5123tc exhibited only 3% of the specific activity of that from normal liver cells. Graham (14) demonstrated that $(Na^+ + K^+)$-ATPase activity of microsomes from normal BHK fibroblasts was 20-fold more active than that from transformed BHK cells. In the present studies, $(Na^+ + K^+)$-ATPase was purified from human kidney, normal and tumor tissues, from the same individual; the activity at various stages of purification is summarized in Table 1. The specific activity increased 8-10 fold in the purification to 100.0 and 16.6 μmol Pi/mg/h from normal and tumor tissues, respectively.

There was no difference in the sodium dodecylsulfate-polyacrylamide gel patterns of the purified enzyme from normal and tumor kidney. Both gave two major bands corresponding to the catalytic subunit and glycoprotein subunits

TABLE 1

Purification of $(Na^+ + K^+)$-Stimulated ATPase From Human Kidney

STEP	Normal		Adenocarcinoma	
	Total Protein (mg)	Specific Activity (μmol Pi/mg/h)	Total Protein (mg)	Specific Activity (μmol Pi/mg/h)
Membranes	84.8	10.4 ± 0.3	25.6	1.9 ± 0.5
NaI-treated membranes	56.4	39.5 ± 1.7	14.2	5.4 ± 0.6
SDS-Solubilized enzyme	6.8	100.0 ± 4.8	2.2	16.6 ± 2.1

Purification procedure is described under "Experimental Procedures". Enzyme assay medium contained, in a total volume of 1.0 ml, 3mM ATP, 100 mM NaCl, 20mM KCl, 4mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.0). Incubation was conducted at 37°C for 10-15 min. with or without ouabain (0.1mM). $(Na^+ + K^+)$-ATPase activity was taken to be the difference in activity with and without ouabain.

of $(Na^+ + K^+)$-ATPase, comparable to the human kidney pure enzyme obtained by Braughler and Corder (6). The results, summarized in Table 2, demonstrate that the $(Na^+ + K^+)$-ATPase purified from human kidney adenocarcinoma was significantly inhibited by macromomycin, largomycin and NSC 327459 (70 to 90% of the enzyme activity being inhibited by 50 μg/ml of the antitumor proteins), where as auromomycin had no effect. Macromomycin and auromomycin produced no inhibition of the enzyme from normal kidney which was, however, inhibited 15-20% by largomycin and NSC 327459.

Preliminary results from kinetic studies indicated that the Km for the $(Na^+ + K^+)$-ATPase from the tumor was less than that for the normal enzyme, which may suggest a subtle alteration in the enzyme structure due to molecular variation in the enzyme protein or by a change in lipid environment in the close association with the enzyme. It has been shown that the activity of $(Na^+ + K^+)$-ATPase is influenced by the type of phospholipid present (2), and also that there are differences in phospholipids and glycolipids in normal and tumor cells (15). For lack of tumor tissue this latter point could not be

TABLE 2

Inhibition of $(Na^+ + K^+)$-ATPase by Antitumor Proteins

Inhibitor	Conc. (μg/ml)	$(Na^+ + K^+)$-ATPase Activity (μmol Pi/mg/h)		Inhibition (%)	
		Normal	Adenocarcinoma	Normal	Adenocarcinoma
Control	-	96.8	18.0	-	-
Macromomycin	10	96.8	10.2	0.0	43.3
	50	96.8	4.2	0.0	76.7
Auromomycin	10	96.8	18.0	0.0	0.0
	50	96.8	17.4	0.0	3.4
Largomycin	10	88.8	9.0	8.3	50.0
	50	80.8	1.2	16.5	93.4
NSC 327459	10	84.8	7.2	12.4	60.0
	50	74.4	4.8	23.2	73.4

The $(Na^+ + K^+)$-ATPase assay system was described in Table 1. The enzyme preparations were preincubated with various amounts of antitumor agents for 5 min. at 37°C. The enzyme reaction, at 37°C for 10-15 min., was conducted with and without ouabain (0.1mM) and terminated by the addition of 0.5 ml of 20% trichloroacetic acid. Inorganic phosphate was determined in 0.5 ml aliquots of the clear supernatant.

pursued, but the possibility exists that the extent of phosphorylation may explain the difference in the specific activities of the tumor and normal human kidney $(Na^+ + K^+)$-ATPase and their sensitivities to macromomycin. The phosphorylation of each $(Na^+ + K^+)$-ATPase by skeletal muscle protein kinase demonstrated that the activities of both enzymes was reduced. The results, summarized in Table 3, indicate that the rate of phosphorylation of both enzymes was stimulated by cAMP, a six fold increase being noted for the enzyme from normal tissue compared to a three-fold increase for the tumor enzyme; the final specific radioactivity was similar for each enzyme. Phosphorylation significantly reduced the enzyme activities (Table 4); the phospho-normal $(Na^+ + K^+)$-ATPase was 30% of the original activity and was now more analogous to the unphosphorylated tumor enzyme. The similarity was further extended by the fact that the phospho-normal enzyme was inhibited 40% by macro-

TABLE 3

Phosphorylation of $(Na^+ + K^+)$-ATPase by Protein Kinase

Source of $(Na^+ + K^+)$-ATPase	^{32}P-incorporation (pmol ^{32}P/mg/min)		
	-cAMP	+cAMP	(+cAMP) - (-cAMP)
Normal Kidney	8.2 ± 0.3	48.4 ± 0.5	40.2 ± 0.5
Adenocarcinoma	14.4 ± 1.1	44.2 ± 3.2	39.8 ± 3.2

Phosphorylation of $(Na^+ + K^+)$-ATPase from human kidney (normal and tumor), was carried out for 5 min. at 37° in a total volume of 100 µl containing 50 mM sodium acetate (pH 6.2), 1mM [γ-^{32}P]ATP (50-70 cmp per pmole), 5mM $MgCl_2$, 6 µg protein kinase from rabbit-skeletal muscle, 100 µg $(Na^+ + K^+)$-ATPase protein and 5µM cyclic AMP, when present. The reaction was started by addition of ATP as described in "Experimental Procedures".

momycin as was the unphosphorylated tumor enzyme. The phospho-tumor enzyme was even further inhibited by macromomycin.

In a recent report, Spector et.al. (16) have demonstrated that the glycoprotein subunit of $(Na^+ + K^+)$-ATPase from Ehrlich ascites tumor cells was

TABLE 4

$(Na^+ + K^+)$-ATPase Activity of Phosphorylated Enzyme and Effect of Macromomycin

Enzyme	Macromomycin (10µg/ml)	$(Na^+ + K^+)$-ATPase activity (µmol Pi/mg/h)	% Control
Human Kidney (normal)			
Control	-	93.1	-
Phosphorylated	-	26.8	28.8
Control	+	93.1	100.0
Phosphorylated	+	16.3	17.5
Human Kidney (adenocarcinoma)			
Control	-	15.1	-
Phosphorylated	-	2.9	19.1
Control	+	8.5	56.3
Phosphorylated	+	1.4	10.0

Reaction conditions were as described in "Experimental Procedures".

phosphorylated by a membrane bound protein kinase at a tyrosyl residue, resulting in reduced efficiency of the enzyme. The phosphorylation of the human $(Na^+ + K^+)$-ATPases by muscle protein kinase resulted in phosphorylation of the glycoprotein subunit, as detected by autoradiography, at a seryl residue; it is seen that here too the phosphate residue reduces the specific activity.

Earlier studies on the action of macromomycin on $(Na^+ + K^+)$-ATPase from dog kidney demonstrated that the inhibitory effect was mediated through the non-catalytic glycoprotein subunit (1). Since macromomomycin inhibits more strongly the phosphorylated $(Na^+ + K^+)$-ATPase of human kidney, it is possible that the sensitivity of a $(Na^+ + K^+)$-ATPase to inhibition by macromomycin is indicative of a phosphorylated glycoprotein subunit, the role of which having been hitherto little understood.

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