Catalytic Function of Nongastric H,K-ATPase Expressed in Sf-21 Insect Cells†

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ABSTRACT: We previously demonstrated that the α−subunit of human nongastric H,K-ATPase (Atp1al1) can assemble with the gastric H,K-ATPase β-subunit (βHK) into an active ion pump upon coexpression in *Xenopus* oocytes. To gain insight into enzymatic functions, we have analyzed the Atp1al1−βHK complex using a baculovirus expression system. The efficient formation of the functional Atp1al1−βHK complex in membranes of Sf-21 insect cells was obtained upon co-infection with recombinant baculoviruses expressing Atp1al1 and βHK. Expression of either protein alone did not produce active ATPase. The effects of K^+, Na^+, pH, and ATP and inhibitors on ATPase activity of the recombinant Atp1al1−βHK complex were analyzed. The Atp1al1−βHK complex was shown to exhibit significant ATPase activity in nominally K^+-free medium. The addition of K^+ stimulated the ATP hydrolysis up to 3-fold with K_m ∼116 μM K^+. The ATPase activity was moderately sensitive to ouabain and to SCH 28080 with apparent K_i values in K^+-free medium of ∼64 μM and ∼93 μM, respectively. Potassium exhibited strong antagonism toward both inhibitors. Assays of the ouabain-sensitive ATPase activity revealed inhibitory effects of Na^+ with the apparent K_i of ∼24 mM in the absence of added K^+ and with K_i within the range of 60−70 mM in the presence of ≥1 mM K^+. Thus, the human nongastric H,K-ATPase represented by the recombinant Atp1al1−βHK complex exhibits enzymatic properties of K^+-dependent ATPase sensitive to ouabain, SCH 28080, and Na^+. It differs from Na,K-ATPase in cation dependence and differs from gastric H,K-ATPase and Na,K-ATPase in sensitivity to inhibitors.

Potassium-dependent animal ATPases (X,K-ATPases) are the most closely related among the various P-type ATPases (*1*). All of the known X,K-ATPases function as cation exchangers which pump K^+ into the cell and Na^+ or H^+ out of the cell, exhibiting a much higher level of sequence homology between their catalytic α-subunits (∼110 kDa) than with other P-ATPases and containing a glycosylated β-subunit (core protein ∼35 kDa) which is absent in other P-ATPases (*1*−*3*). The X,K-ATPase family combines three distinct groups of ion pumps. Two groups, one consisting of the Na,K-ATPase isozymes formed by four α and three β isoforms and the second which includes the gastric H,K-ATPase, have long been known and studied extensively (*2*, *3*). Recently discovered catalytic α-subunits of nongastric H,K-ATPases encoded by the human ATP1AL1 gene and its animal homologues represent the third distinct group (*4*−*6*). Evidence for the existence of ion-transporting P-ATPases, whose functional properties are closely related, but not identical, to that of the Na,K-ATPase or the gastric H,K-ATPase and which are involved in maintenance of electrolyte homeostasis through K^+ absorption and proton secretion in kidney and colon especially in disease processes including ionic and acid−base disorders, was obtained in many previous physiological and pharmacological studies (for review, see refs *7* and *8*). Up to now none of these enzymes has been purified and analyzed directly; therefore, many aspects of structure and functions remain undetermined.

The human ATP1AL1 gene was originally identified as a gene exhibiting a sequence similar to that of the Na,K-ATPase α-subunit cDNA but not corresponding to any ion pumps known at that time. That is why it was designated as an α-like gene (AL1) (*9*, *10*). Further studies have revealed that ATP1AL1 is transcriptionally competent (*11*). The cDNA for ATP1AL1 was cloned from human kidney and skin libraries (*12*), and closely related cDNAs have been cloned from rat, rabbit, and guinea pig distal colons (*13*−*15*) and mouse and dog kidneys (*16*). The encoded human protein Atp1al1[1] and its animal homologues were found to be equally distant structurally (63−64% amino acid sequence identity) from both the Na,K-ATPase α-subunit isoforms and the gastric H,K-ATPase α-subunit (*12*). So far, no specific β-subunit that is an authentic counterpart of the Atp1al1 has been identified.

The first data on the functional properties of Atp1al1 which allowed us to define it as a catalytic subunit of the human nongastric H, K-ATPase were obtained through its heterologous expression in *Xenopus* oocytes in combination with the gastric H,K-ATPase β-subunit (βHK), which was found

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[1] Abbreviations: Atp1al1, a catalytic subunit of nongastric H,K-ATPase encoded by the human ATP1AL1 gene; βHK, β-subunit of the gastric H,K-ATPase; α1NK, α-subunit of the Na,K-ATPase; β1NK, β2NK, or β3NK, isoforms of the β-subunit of the Na,K-ATPase; Sf-9 and Sf-21, *Spodoptera frugiperda* insect cell lines; AcMNPV, wild-type baculovirus; AcAtp1al1 and AcβHK, recombinant baculoviruses expressing Atp1al1 and βHK, respectively; SCH 28080, 2-methyl-8-(phenylmethoxy)imidazo[1,2a]pyridine-3-acetonitrile.

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to be an efficient substitute of the unknown authentic β-subunit (*4*). Inward K^+ transport mediated by the stable Atp1al1−βHK complex in the plasma membrane of oocytes was revealed using an $^{86}Rb^+$ uptake assay. Proton extrusion from cRNA-injected oocytes was detected by measurement of intracellular and external pH. Most significantly, ion transport mediated by the Atp1al1−βHK was found to be sensitive to ouabain and, with low affinity, to the gastric H,K-ATPase inhibitor SCH 28080 (*4*). Similar observations were made when the Atp1al1−βHK complex was expressed in mammalian HEK-293 cells (*17*). Also, it was recently reported that the Atp1al1−βHK complex expressed in HEK-293 cells lowers intracellular Na^+ concentrations, suggesting that transport modes of nongastric ATPase may not be limited to H^+/K^+ exchange and may also include Na^+/K^+ exchange (*18*).

Data outlined above have demonstrated that the Atp1al1 is a constituent of a novel nongastric type of H,K-ATPase which, in contrast to the gastric H,K-ATPase, is sensitive to cardiotonic steroids such as ouabain, which were considered as specific inhibitors of the Na,K-ATPase. Thus, significant differences in structure and sensitivity to inhibitors between gastric and nongastric H,K-ATPases clearly indicate that these enzymes represent separate groups within the family of X,K-ATPases and obviously cannot be classified as isoforms of one enzyme.

Up to now, it remains unclear whether one of the known β-subunits (*19*−*21*) or a hitherto unidentified member of the X,K-ATPase β-subunit family is a real subunit of nongastric H,K-ATPases. The currently available experimental data are rather controversial. Thus, our expression studies revealed that the βHK is capable of assembling with the Atp1al1 more efficiently than other known X,K-ATPase β-subunits (*4*, *22*). However, the βHK is probably not the real counterpart of the Atp1al1 in vivo, because patterns of their tissue-specific expression are different. The presence of βHK mRNA has only been demonstrated in stomach and kidney (for review, see ref *23*), but expression of the ATP1AL1 was detected at significant levels not only in kidney but also in skin and at a lower level in colon, brain, placenta, and lung (*11*, *12*, *16*, *24*). On the other hand, it was recently reported that β1NK can also form a functional ATPase complex with the α-subunit of rat nongastric, colonic H,K-ATPase expressed in *Xenopus* oocytes (*25*). In other recent studies, β1NK (*26*, *27*) or β3NK (*28*) were named as a real β-subunit of rat nongastric H,K-ATPase, because corresponding antibodies recognized the protein which co-immunoprecipitated with the nongastric H,K-ATPase α-subunit from rat colon or kidney microsomes. However, unless these immunochemical data are confirmed by amino acid sequencing, it cannot be excluded that the antibodies used only cross-react with the unknown β-subunit sharing common epitope(s) with β1NK or β3NK. In our opinion, none of the known β subunits but rather a hitherto unidentified β-subunit with structural features resembling that of βHK serves as a real counterpart of Atp1al1 in vivo (*22*).

This study was designed to develop the approach for a detailed characterization of enzymatic properties of the human nongastric H,K-ATPase, which until the discovery of the authentic β-subunit is represented by the recombinant Atp1al1−βHK complex. On the basis of previous experience (*29*), the baculovirus expression system was selected for these purposes, which we consider a convenient way for preparation of the membrane-bound ATPase complex in amounts sufficient for extensive biochemical studies. The efficient expression of the functionally active recombinant Atp1al1−βHK complex was obtained using *Spodoptera frugiperda* (Sf-21) insect cells for infection. Membrane preparations of these cells were used for the first time to measure the principal Atp1al1−βHK catalytic functions. A preliminary account of part of this work has been presented (*30*, *31*).

EXPERIMENTAL PROCEDURES

DNA Constructs. Baculovirus transfer vectors were constructed using the p2Bac vector (Invitrogen). The entire coding region of the ATP1AL1 cDNA (including 44 bp of 5′- and 118 bp of 3′-untranslated regions) was excised with *Bam*HI from the pHAS 34.1 clone (in the vector pSP64) isolated from a human skin cDNA library (*12*) and cloned into the p2Bac vector under the polyhedrin promoter at the *Bam*HI site to produce p2Bac-Atp1al1. The cDNA clone of the rabbit gastric H,K-ATPase β-subunit (kindly provided by Dr. George Sachs, UCLA) was digested with *Eco*RI. The 1366 bp fragment containing a coding region plus 53 bp of 5′- and 438 bp of 3′-untranslated sequences was isolated, blunt-ended, and subcloned into p2Bac vector under the p10 promoter at the *Stu*I site to produce p2Bac-βHK. The structures of both plasmids were verified by extensive restriction mapping and DNA sequencing.

Isolation of Recombinant Viruses. All the procedures involving insect Sf-9 and Sf-21 cells and baculoviruses were performed according to standard protocols (*32*) as described before (*29*). Sf-9 cells (Invitrogen) were grown in monolayer or suspension cultures at 27 °C in TNM-FH medium (JRH Biosciences) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. To produce recombinant viruses, Sf-9 cells were cotransfected with transfer vectors (p2Bac-Atp1al1 and p2Bac-βHK) and linearized wild-type AcMNPV viral DNA using the Linear Transfection Module (Invitrogen). Recombinant baculoviruses were identified as occlusion-negative plaques and purified by at least three rounds of plaque purification. The ability of recombinant baculoviruses to express desired proteins was confirmed by immunoblot analysis (described below). Stocks of purified recombinant viruses, AcAtp1al1and AcβHK, were stored at 4 °C. During these studies, we noted that Sf-9 cells are more efficient than Sf-21 cells for the purpose of amplification of recombinant viruses. In contrast, Sf-21 cells were found to be more efficient than Sf-9 cells in respect of formation of the functional Atp1al1−βHK complex.

Expression of the Atp1al1−βHK Complex in Sf-21 Cells. Insect Sf-21 cells (Invitrogen) were grown in a 250 mL spinner flask at room temperature in 100 mL of Grace's insect medium (JRH Biosciences) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. For infection, log-phase high viability Sf-21 cells were plated into 75 cm^2 tissue culture flasks at a density of 10^7 cells per flask and allowed to attach and grow overnight at 27 °C. Infection with recombinant baculoviruses was done in fresh medium containing (in addition to the above) 1% (v/v) ethanol and 0.1% (w/v) glucose at a multiplicity of infection for each virus of about 10. [Supplementation of the culture media with additional glucose and

ethanol (*33*, *34*) had positive effects on the level of expression of the functional Atp1al1−βHK complex.] Infected Sf-21 cells were incubated at 27 °C for about 72 h before being harvested.

Isolation of Microsomes from Sf-21 Cells. For a typical preparation to be used in enzymatic assays, infected Sf-21 cells were collected from five 75 cm^2 flasks by gentle scraping and low-speed centrifugation (*29*). Cells were resuspended in ice-cold buffer A [30 mM histidine−HCl (pH 6.8), 250 mM sucrose, and 1 mM EDTA] containing a mixture of protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 5 mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 μg/mL pepstatin) and disrupted by sonication using three pulses of 15 s with a Branson Sonifier 250. After centrifugation for 5 min at 500*g* (4 °C) to remove unbroken cells and nuclei, the supernatant was centrifuged for 30 min at 175000*g* (4 °C). The final pellet was washed with buffer A, then resuspended again in the same buffer at a concentration of about 1−1.25 mg/mL, and used for enzymatic activity assays on the same day as outlined below. Using this procedure, the yield of microsomes from five 75 cm^2 flasks of cells infected with the recombinant AcAtp1al11 and AcβHK viruses was about 6−7.5 mg of protein determined by the Lowry method using bovine serum albumin as the standard.

Immunoblot analysis of isolated membrane preparations was performed as previously described (*29*). We have used affinity-purified rabbit polyclonal Atp1al1 antibodies that were raised against a recombinant N-terminal fragment of Atp1al1 (Ser^{14}−Ile^{104}) prepared as described (*35*). The expression of βHK was demonstrated using monoclonal antibody 2G11 (*36*), kindly provided by Dr. John Forte (University of California, Berkeley, CA) or purchased from Affinity BioReagents, Golden, CO. Detection was done using appropriate alkaline phosphatase conjugated secondary antibodies with indoxyl phosphate/nitroblue tetrazolium as substrate.

ATPase Assays. ATPase activity of the recombinant Atp1al1−βHK complex in microsomes isolated from infected Sf-21 cells and control membrane samples was assayed by measuring the initial rate of release of $^{32}P_i$ from [γ-^{32}P]ATP essentially as described before (*29*). In a typical experiment, the ATPase activity of samples (100 μg of total protein) was measured in a final volume of 1 mL of assay solution containing 2 mM Tris−[γ-^{32}P]ATP, 3 mM $MgCl_2$, 1 mM Tris−EGTA, and 20 mM Tris-HCl (pH 7.4) at 37 °C for 30 min. Prior to the addition of ATP, the reaction mixture was preincubated with 2 μM or 2 mM ouabain or without inhibitor for 20 min at 37 °C. Either 1 mM NaN_3 or 1 mM KN_3 or oligomycin (5 μg/mL), to which the Atp1al1−βHK ATPase activity is insensitive, was included in the reaction medium to inhibit the endogenous mitochondrial ATPase activity. Potassium azide was kindly provided by Dr. Mitsuru Kubota (Harvey Mudd College, Claremont, CA). Additional components presented in particular reaction mixtures are listed in the corresponding figure legend. The amount of hydrolyzed ATP never exceeded 10% of the total ATP present in the sample.

The specific activity of the recombinant Atp1al1−βHK complex was usually determined as ATP hydrolysis in the presence of 2 μM ouabain. This concentration of ouabain completely inhibits endogenous ouabain-sensitive ATPase activity present in Sf-21 cell membranes but has very little effect on the Atp1al1−βHK ATPase activity. The background level of ATP hydrolysis was determined after preincubation of the samples with 2 mM ouabain. Prior to use, the specific activity of each preparation of the recombinant Atp1al1−βHK complex was determined as the difference between activities measured in the presence of 2 μM and 2 mM ouabain. Specific ATPase activity was measured in the nominally K^+-free medium and varied in different preparations from 0.4 to 1.65 μmol mg^{-1} h^{-1}. The ratio of the ouabain-sensitive activities of the recombinant complex vs the endogenous activity of the Sf-21 cell membranes also varied in the range from 3 to almost 10 in the best preparations. The ATP hydrolysis rate was usually stimulated 1.5−3-fold by the addition of K^+ into the assay medium.

When the K_m of a substrate or an activator was to be determined, reaction mixtures had the same compositions as indicated above, except for the varying concentration of the indicated ligand. In the absence of added KCl, the K^+ concentration in the reaction medium was about 5 μM as was determined by means of atomic absorption on an atomic absorption spectrophotometer (Perkin-Elmer, Model 5000). To determine the effect of different ouabain or SCH 28080 concentrations on the ATP-hydrolyzing activity of the recombinant Atp1al1−βHK complex, samples were preincubated with the indicated concentrations of inhibitor for 20 min at 37 °C prior to the addition of ATP. SCH 28080 (kindly provided by Dr. George Sachs, UCLA, and by Dr. Sabbarao Vemulapalli, Schering Plough Research Institute) was added from a stock solution in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the assay solution was no more than 1%, which had no significant effect on the ATPase activity of the Atp1al1−βHK complex.

Control experiments were done with membrane samples isolated from noninfected Sf-21 cells or from Sf-21 cells infected either with wild-type AcMNPV baculovirus or with the recombinant viruses AcAtp1al11 or AcβHK.

As a rule, at least three independent representative experiments with triplicate determinations for each particular condition were performed to determine each functional parameter of the Atp1al1−βHK ATPase complex reported here. The unpaired Student's *t*-test was used to estimate levels of significance. $P < 0.05$ was considered significant. Analysis of kinetic data was done using the KaleidaGraph software package. The apparent K_m values were calculated through fitting of the experimental data by nonlinear regression analysis to the Michaelis−Menten equation. Curve fitting to single- or two-site models was used to calculate K_i of inhibition by ouabain, SCH 28080, and sodium.

RESULTS

Atp1al1 and βHK Protein Expression in Insect Cells. Based on our earlier studies on the expression of the Atp1al1−βHK complex in *Xenopus* oocytes (*4*), it was reasonable to expect that the rabbit βHK will also be assembled with the human Atp1al1 in the insect cell membrane upon baculovirus-driven coexpression. Recombinant baculoviruses (the AcAtp1al11, containing cDNA coding for the Atp1al1 protein under the AcNPV polyhedrin promoter; the AcβHK, containing cDNA of βHK under p10

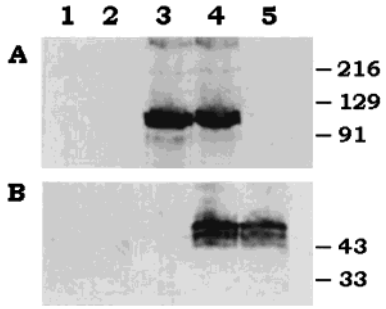

FIGURE 1: Immunoblot analysis of the Atp1al1 and βHK coexpressed in insect Sf-21 cells. Two blots were prepared from electrophoretically separated samples of cell membranes isolated from noninfected Sf-21 cells (lane 1), from cells infected with the wild-type AcMNPV baculovirus (lane 2), from cells infected with the recombinant baculovirus, AcAtp1al1 (lane 3), from cells co-infected with AcAtp1al1 and AcβHK (lane 4), and from cells infected with AcβHK (lane 5). Each lane contains 10 μg of membrane protein. Blots were probed with Atp1al1-specific polyclonal antibodies (A) or with the βHK-specific 2G11 monoclonal antibody (B) as described in Experimental Procedures. The positions of the molecular mass markers (kDa) are shown to the right.

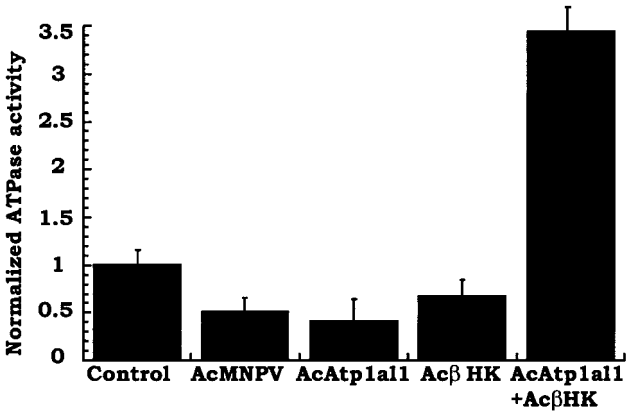

FIGURE 2: Ouabain-sensitive ATPase activity of membrane preparations from Sf-21 cells expressing the Atp1al1 and βHK. Microsomes were isolated from Sf-21 cells which were noninfected or infected with the wild-type AcMNPV baculovirus and from Sf-21 cells infected with the recombinant baculoviruses AcAtp1al1 and AcβHK alone or in combination. The ATPase activity of the isolated samples was assayed after preincubation with or without 1 mM ouabain as described under Experimental Procedures in the presence of 1 mM K^+ and 1 mM NaN_3. Ouabain-sensitive components of ATPase activity of each preparation were determined in triplicate, normalized to activity of membranes from noninfected Sf-21 cells, and expressed as the mean $\pm$ SE. Data shown correspond to one out of four independent representative experiments, in which specific activity of control uninfected cells was 0.15 μmol mg^{-1} h^{-1}.

promoter) were generated for this study using the transfer vector, p2Bac. We compared the capabilities of two insect cell lines (Sf-9 and Sf-21) infected with the AcAtp1al1 and/or the AcβHK baculoviruses to synthesize recombinant proteins.

The expression of the virally induced proteins was determined by immunoblots of membrane fractions isolated from infected cells using antibodies specific to the human Atp1al1 (*35*) and to the gastric H,K-ATPase β-subunit (*36*). Figure 1 represents results obtained in experiments with Sf-21 cells infected with the AcAtp1al1 (lanes 3A and 3B) or with the AcβHK (lanes 5A and 5B) or co-infected with both AcAtp1al1 and AcβHK (lanes 4A and 4B). The Atp1al1-specific antibodies (Figure 1A) revealed a protein band (lanes 3A and 4A) whose mobility correlates well with the molecular mass of about 115 kDa, as was expected from the deduced amino acid sequence (*4*, *12*, *35*). It was previously shown that insect cells usually produce X,K-ATPase β-polypeptides as a mixture of species whose glycosylation states are different (*29*, *37*, *38*). Similarly, the βHK-specific antibody 2G11 revealed several immunoreactive bands migrating in the area from 43 to 57 kDa (lanes 4B and 5B). Evidently, these bands represent βHK polypeptides containing different numbers of N-linked carbohydrate chains with structures typical for insect cells (*32*). The antibodies used did not cross-react with other proteins present in membranes isolated from control Sf-21 cells which were noninfected (lanes 1A and 1B) or infected with the wild-type AcMNPV baculovirus (lanes 2A and 2B). Considered together, these results clearly demonstrated that both desired proteins, Atp1al1 and βHK, are present in membranes of Sf-21 cells co-infected with AcAtp1al1 and AcβHK viruses (lanes 4A and 4B). Practically the same pictures were observed upon immunoblotting of membrane fractions isolated from Sf-9 cells infected with the AcAtp1al1 and/or the AcβHK viruses (data not shown).

Detection of the Functionally Active Recombinant Atp1al1−βHK Complex. It is well established that participation of both α- and β-subunits is absolutely required for the formation of functionally active X,K-ATPase (*2*−*6*, *19*, *21*, *29*, *30*). To determine whether the coexpression of Atp1al1 and βHK results in the formation of a functionally active ATPase complex, membrane preparations from Sf-9 and Sf-21 insect cells noninfected or infected with the wild-type or recombinant viruses expressing Atp1al1 and βHK were assayed for ouabain-sensitive ATPase activity. Figure 2 illustrates the principal results obtained with Sf-21 cells. The membranes from noninfected Sf-21 insect cells clearly contained some ouabain-sensitive ATPase activity. The level of this endogenous ouabain-sensitive ATPase activity was small and varied in different preparations from $\leq$0.03 to 0.15 μmol mg^{-1} h^{-1}. In this respect, Sf-21 cells are similar to previously studied Sf-9 cells whose membranes also exhibit small amounts of ouabain-sensitive ATPase activity (*29*, *37*, *38*). The ouabain-sensitive activities of membranes from Sf 21 cells infected with the wild-type AcMNPV baculovirus or with one of the baculoviruses expressing either the Atp1al1 or the βHK alone did not differ significantly from that of membranes from noninfected cells, thus indicating that in all these cases virally induced proteins did not exhibit ATPase activity. The same observations were made when the above set of experiments was performed using Sf-9 insect cells (data not shown). Simultaneous expression of Atp1al1 and βHK proteins in membranes of Sf-9 cells co-infected with AcAtp1al1 and AcβHK viruses also did not lead to a substantial increase in specific ouabain-sensitive ATPase activity (data not shown). A significant increase of specific ouabain-sensitive ATPase activity was detected in the membranes of Sf-21 cells that were co-infected with AcAtp1al1 and AcβHK viruses (Figure 2). This experiment demonstrated that the Atp1al1 and the βHK are indeed capable of assembling into a functional ATPase complex upon coexpression in Sf-21 insect cells.

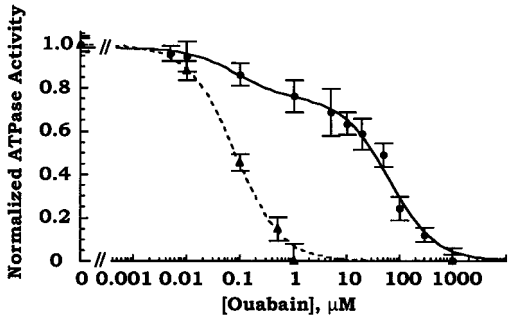

FIGURE 3: Ouabain inhibition of ATP hydrolysis by the recombinant Atp1al1−βHK complex (●) and by control Sf-21 cell membranes (▲). ATPase activity was assayed after preincubation in the presence of indicated concentrations of ouabain in assay medium containing 1 mM NaN_3 and no added KCl. Data from three independent experiments were normalized to the activity in the absence of ouabain and expressed as the mean ± SE. Under these conditions, maximal specific activities of the samples of the Atp1al1−βHK complex used were in the range of 0.68−0.83 μmol mg^{-1} h^{-1}. The endogenous ATPase activities of Sf-21 cells in the absence of ouabain were in the range of 0.12−0.15 μmol mg^{-1} h^{-1}. Curve fitting to single- **(▲)** or two-site **(●)** models was done using KaleidaGraph software.

Ouabain Sensitivity of the Recombinant Atp1al1−βHK Complex. To define conditions that will allow us to more precisely discriminate ATP hydrolysis by the Atp1al1−βHK complex from that by the endogenous insect ATPase, we compared the ouabain sensitivity of corresponding membrane preparations (Figure 3). In preliminary experiments, we found out that ouabain-sensitive ATPase activity of the expressed complex can be detected in the absence of added K^+ in nominally K^+-free medium. In this case, the K^+ concentration in the reaction medium was about 5 μM as was determined by means of atomic absorption. To avoid the effects of potential ouabain−potassium antagonism, this nominally K^+-free medium was used to detect the formation of the ouabain-sensitive Atp1al1−βHK complex in membrane preparations of infected Sf-21 cells. As shown in Figure 3, ouabain inhibits ATPase activity of control Sf-21 cell membranes and preparation of the expressed Atp1al1−βHK complex in a dose-dependent manner. The endogenous ouabain-sensitive ATPase activity of control Sf-21 cells is inhibited by ouabain with an apparent K_i of 79 ± 12 nM. It should be pointed out that in this set of experiments the endogenous ATPase activity in the absence of ouabain comprised about 20% of that of the Atp1al1−βHK complex. The endogenous ATPase activity of Sf-21 cells corresponds to the upper portion of the biphasic curve observed in the case of the Atp1al1−βHK-containing membranes. The lower part of the curve represents the ATPase activity of the recombinant Atp1al1−βHK complex in nominally K^+-free medium, which is inhibited by ouabain with an apparent K_i of 64 ± 10 μM (Figure 3).

The endogenous ouabain-sensitive ATPase activity of Sf-21 cells was almost totally inhibited by 1 μM ouabain. To ensure complete abolishment of this intrinsic ATPase activity, the specific activity of the Atp1al1−βHK complex was usually determined in further assays as ATP hydrolysis in the presence of 2 μM ouabain or as the difference between activities measured in the presence of 2 μM and 2 mM ouabain. Specific activities of the recombinant Atp1al1−βHK ATPase preparations used in the current study were no less than 0.4 μmol mg^{-1} h^{-1} in nominally K^+-free medium.

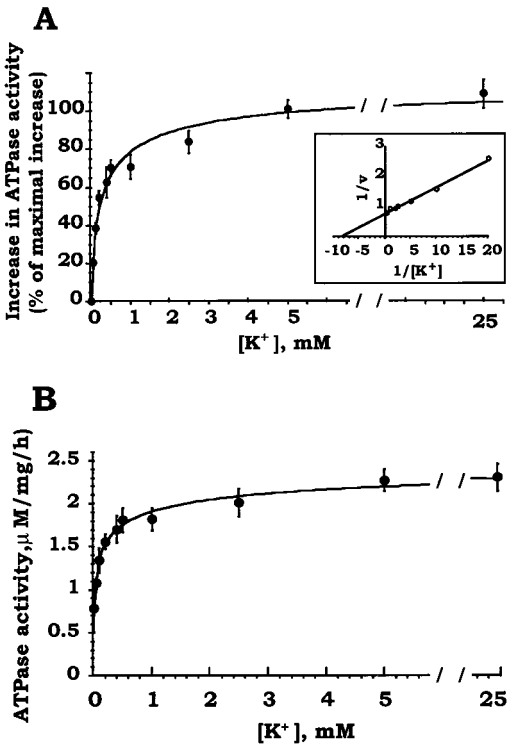

FIGURE 4: Ouabain-sensitive ATPase activity of the recombinant Atp1al1−βHK complex as a function of K^+ concentration. ATPase activity of membrane preparations from Sf-21 cells expressing the Atp1al1−βHK complex was determined at the indicated concentrations of added KCl in three independent representative experiments with triplicate determinations for each condition in the presence of ouabain (2 μM) and oligomycin (5 μg/mL). (A) Data representing effects of added K^+ concentration are expressed as the percent of the maximal increase of the ATPase activity over the activity in nominally K^+-free medium. Each value represents the mean ± SE of three independent experiments. Inset: Lineweaver−Burk plot of the increase of the ATP hydrolysis rate (μmol mg^{-1} h^{-1}) vs concentration of added K^+ (0.05−2.5 mM). K_m = 116.5 μM. Maximal increase in velocity = 1.31 μmol mg^{-1} h^{-1}. (B) Data from one of three independent experiments including part of the specific activity determined in nominally K^+-free medium. Specific activities were determined in triplicate and shown as the mean ± SE.

The Atp1al1−βHK complex was found to be sensitive to vanadate like all other known P-type ATPases (*1*−*3*); this inhibitor in 100 μM concentration completely abolished Atp1al1−βHK ATPase activity (data not shown). The Atp1al1−βHK ATPase activity was found to be resistant to oligomycin (5 μg/mL) and 1 mM NaN_3 or KN_3 (data not shown). Therefore, either oligomycin or one of the azides was present in any assay medium to reduce the background level of the ATPase activity of insect cell membranes that primarily originated from mitochondrial ATPase. In this respect, the properties of all three reagents at the concentrations shown above are practically the same.

K^+ Dependence of the Atp1al1−βHK Complex. Experiments aimed to assess the ouabain-sensitive ATPase activity of the Atp1al1−βHK complex as a function of K^+ concentration are described in Figure 4. Data shown in Figure 4A represent only effects of added potassium on ouabain-sensitive ATPase activity of the Atp1al1−βHK complex, disregarding the activity that this complex exhibits in nominally K^+ -free medium. The specific ATPase activity

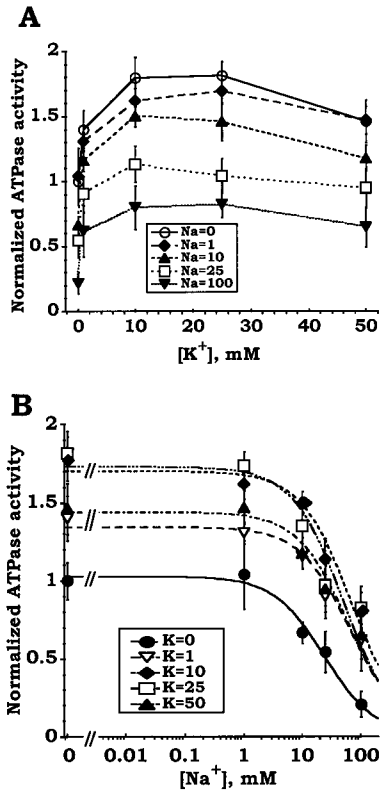

FIGURE 5: ATP hydrolysis by the Atp1al11−βHK complex at different K^+ plus Na^+ concentrations. ATPase activity of membrane preparations from Sf-21 cells expressing the Atp1al1−βHK complex was determined at the indicated concentrations of added K^+ and/or Na^+ in the presence of ouabain (2 μM) and oligomycin (5 μg/mL). Background ATPase activities of samples were determined after preincubation with 2 mM ouabain. Data from one of three independent representative experiments that produced similar results are presented. Specific activities above background were determined in triplicate for each condition, normalized to the activity in nominally K^+- and Na^+-free medium (0.65 μmol mg^{-1} h^{-1}), and expressed as the mean ± SE. Data are plotted as a function of varying $[K^+]$ at constant $[Na^+]$ (A) or as a function of varying $[Na^+]$ at constant $[K^+]$ (B). Curve fitting to a single-site model was done using KaleidaGraph software.

was stimulated with added K^+ in a concentration-dependent manner and flattened out in the 5−25 mM range. The apparent K_m of the Atp1al1−βHK complex for ATP hydrolysis activation by added K^+ in the concentration range of 0.05−2.5 mM estimated from the Lineweaver−Burk plot was 116.5 μM (Figure 4A, inset). Specific activities of the samples of the Atp1al1−βHK complex used in this set of experiments were in the range of 0.63−0.79 μmol mg^{-1} h^{-1} in the absence of added K^+. To exemplify the relation between K^+ -sensitive and insensitive components of the ouabain-sensitive ATPase activity of the Atp1al1−βHK complex, one set of experimental data has been replotted in Figure 4B including the specific activity detected in nominally K^+-free medium. In this set of experiments the addition of K^+ to the assay medium resulted in about 3-fold stimulation of the specific ATPase activity of the Atp1al1−βHK complex. The magnitude of the maximal K^+ stimulation of the Atp1al1−βHK complex varied in different preparations of Sf-21 cell membranes from 1.5- to 3.4-fold.

Inhibitory Effect of Na^+ on ATP Hydrolysis by the Atp1al1−βHK Complex. The experiments of Figure 5 were designed to gain explicit information about the effects of monovalent cations on ATPase activity of the Atp1al1−βHK complex. For each set of these experiments, the individual preparation of the Atp1al1−βHK complex in Sf-21 cell membranes was first carefully washed to remove K^+ and Na^+ ions, and then the ouabain-sensitive ATPase activity of this nominally K^+- and Na^+-free sample was measured in assay solutions containing added KCl and/or NaCl in concentrations which varied from 0 to 50 mM for K^+ and from 0 to 100 mM for Na^+. Specific activities of the samples of the Atp1al1−βHK complex used in this set of experiments were in the range of 0.53−0.74 μmol mg^{-1} h^{-1} in the absence of added K^+. Plots in Figure 5A represent ATPase activity as a function of varying K^+ concentrations at constant Na^+ concentrations. To simplify presentation, specific activities were normalized to that in nominally K^+- and Na^+-free medium. As shown in this figure, an increase in added K^+ concentrations in the low (1−10 mM) range stimulated the specific ATPase activity of the Atp1al1−βHK complex at all Na^+ concentrations tested. The rate of ATP hydrolysis did not change significantly within the range of 10−25 mM K^+ and slightly decreased at 50 mM K^+. It is important to note that the Atp1al1−βHK complex exhibited the highest level of ATPase activity in nominally Na^+-free medium and an increase in Na^+ concentration in the assay medium led to a significant decrease in the ATP hydrolysis rate at all K^+ concentrations tested. [These effects of monovalent cations on ATPase activity of the Atp1al1−βHK complex were not detected in earlier studies (*30*, *31*) due to relatively low specific ATPase activity of preparations used and insufficient removal of sodium or potassium ions from the samples.]

To illustrate more clearly the effects of added Na^+ on ATPase activity, experimental data have been replotted as a function of varying Na^+ concentrations at constant $[K^+]$ (Figure 5B). Na^+ exhibited an inhibitory effect on ATP hydrolysis by the Atp1al1−βHK complex at all K^+ concentrations tested. This effect was the most pronounced in nominally K^+-free medium when Na^+ alone was added to assay solutions. The apparent K_i for these conditions was calculated to be 24.3 ± 4.2 mM. Na^+ inhibition of the Atp1al1−βHK complex was less pronounced in the presence of added K^+, and already at 1 mM K^+ the value of the apparent K_i for Na^+ inhibition increases about three times (68.4 ± 13.6 mM). A further increase in K^+ concentration does not significantly affect Na^+ inhibition, and the apparent K_i values were calculated to be 70.6 ± 17.2 mM at 10 mM K^+, 66.4 ± 20.8 mM at 25 mM K^+, and 62.3 ± 13.8 mM at 50 mM K^+. Thus, results of these experiments clearly demonstrated that ATP hydrolysis by the Atp1al11−βHK complex is sodium sensitive, and the inhibitory effect of Na^+ is most noticeable in K^+-free medium.

pH Sensitivity and ATP Dependence of ATPase Activity of the Atp1al1−βHK Complex. The influence of pH in assay medium on the ouabain-sensitive ATPase activity of the Atp1al1−βHK complex within the range of pH values from 5.6 to 8.0 was analyzed in the experiments presented in Figure 6. It was shown that specific ATPase activity of the Atp1al1−βHK was markedly affected by pH. It sharply increased with increased pH, reached the maximum at pH ~7.5, and then decreased at higher alkaline pH. On the basis of these observations, we assumed that the pH within the 7.2−7.8 range was optimal for the Atp1al1−βHK ATPase

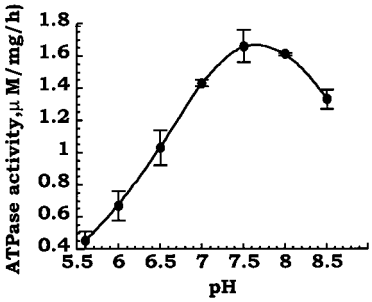

FIGURE 6: Ouabain-sensitive ATPase activity of the Atp1al1−βHK complex at different pH values. ATP hydrolysis by membrane preparations of the Atp1al1−βHK complex was measured in 5 mM PIPES buffer adjusted with Tris to the indicated pH, in the presence of 1 mM KCl, 1 mM NaN_3, and 2 μM ouabain. Background ATPase activities of samples were determined after preincubation with 2 mM ouabain. Data from one of three independent representative experiments that produced similar results are presented. Specific activities above background were determined in triplicate for each condition and expressed as the mean ± SE.

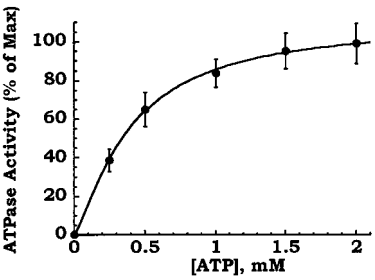

FIGURE 7: Ouabain-sensitive ATPase activity of the Atp1al1−βHK complex as a function of ATP concentration. Ouabain-sensitive ATPase activity of microsomes from Sf-21 cells expressing the Atp1al1−βHK complex was determined at the indicated ATP concentrations in the presence of 1 mM KCl, 1 mM NaN_3, and 2 μM ouabain with triplicate determinations for each condition. Background ATPase activities of samples were determined after preincubation with 2 mM ouabain. Data are expressed as the percent of the maximal ATPase activity observed (0.68−0.85 μmol mg^{-1} h^{-1}). Each value represents the mean ± SE of three independent representative experiments.

activity assays. Data shown in Figure 6 were determined in the presence of 1 mM K^+. When the same set of measurements was performed in the presence of 10 mM K^+, the shape of the pH curve did not change significantly, although a slightly higher level of activity was observed at each indicated pH (data not shown). The "bell curve" of the pH dependence exhibited by the ATPase activity of the Atp1al1−βHK complex is a typical feature of other known X,K-ATPases, the gastric H,K-ATPase (*39*, *40*) and Na,K-ATPase (*41*).

As shown in Figure 7, measurements of ouabain-sensitive ATPase activity at ATP concentrations varying in the low millimolar range in the presence of 1 mM K^+ demonstrated that the Atp1al1−βHK complex displayed ATP dependence with an apparent K_m of 0.39 ± 0.03 mM. This value is in agreement with apparent K_m values for the interaction of ATP at low-affinity binding sites of the native gastric H,K-ATPase (*39*, *42*, *43*) and native and recombinant Na,K-ATPase (*29*, *37*, *38*).

Thus, parameters of the ATPase activity of the Atp1al1−βHK complex with respect to pH and ATP dependencies are within the same range of values as those described previously for other X,K-ATPases.

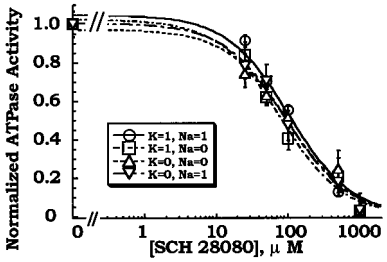

FIGURE 8: SCH 28080 inhibition of ATP hydrolysis by the recombinant Atp1al1−βHK complex. ATPase activity was assayed after preincubation at the indicated concentrations of SCH 28080 and 2 μM ouabain in the nominally K^+- and Na^+-free medium containing oligomycin (5 μg/mL) (△) or in media containing either 1 mM NaN_3 (▽) or 1 mM KN_3 (□) or 1 mM KN_3 and 1 mM NaCl (○). Background ATPase activities of samples were determined after preincubation with 2 mM ouabain. Values of specific activities determined in triplicate for each condition were normalized to the activity in the absence of SCH 28080 and expressed as the mean ± SE. Each curve corresponds to one representative from three to five independent experiments that produced similar results. Curve fitting to a single-site model was done using KaleidaGraph software.

Sensitivity of the Atp1al1−βHK Complex to SCH 28080 and K^+ Antagonism toward SCH 28080 and Ouabain. It was shown in previous studies that Rb^+ uptake mediated by the Atp1al1−βHK complex expressed in *Xenopus* oocytes (*4*) or in HEK-293 cells (*17*) is moderately sensitive to SCH 28080, a K^+-competitive inhibitor of the gastric H,K-ATPase (*3*, *44*−*46*). In the current study we analyzed the effects of SCH 28080 on ATP hydrolysis by the Atp1al1−βHK complex. Specific activities of the samples tested in these experiments were in the range of 0.78−1.64 μmol mg^{-1} h^{-1} in the absence of added K^+. In preliminary experiments, we determined that the endogenous ouabain-sensitive ATPase activity presented in Sf-21 cell membranes is not sensitive to SCH28080 (data not shown).

To exclude the influence of potential SCH 28080−potassium antagonism, the first set of experiments was performed in nominally K^+- and Na^+-free medium containing oligomycin. As shown in Figure 8, SCH 28080 inhibits ATPase activity under these conditions in a dose-dependent manner. At a concentration of 1 mM, this compound practically abolished the specific ATPase activity of the Atp1al1−βHK complex. The residual background ATPase activities of membrane preparations used, which have been determined after preincubation with 2 mM ouabain and/or 1 mM SCH 28080, did not differ significantly (data not shown). The apparent K_i for SCH 28080 was calculated to be 100 ± 23 μM. An increase in K^+ and/or Na^+ concentrations to 1 mM in assay media did not have a significant effect on inhibition of the Atp1al1−βHK complex by SCH 28080. In the presence of 1 mM K^+ (added as KN_3) the apparent K_i was 89 ± 11 μM. Similarly, when ATPase activity was measured in medium containing 1 mM K^+ and 1 mM Na^+ or 1 mM Na^+ alone (added as NaN_3), the apparent K_i values were calculated as 104 ± 22 μM and 94 ± 16 μM, respectively. These values are in good agreement with the apparent K_i of 131 ± 42 μM for SCH 28080 inhibition of Rb^+ uptake by the Atp1al1−βHK complex (*17*), indicating that its ATP-hydrolyzing and ion-transporting functions are equally sensitive to SCH 28080.

Since no competition between potassium and SCH 28080 was detected at a K^+ concentration up to 1 mM, in the next

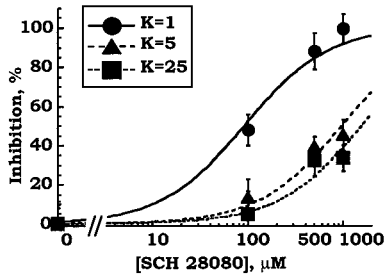

FIGURE 9: Potassium−SCH28080 antagonism. ATPase activity was assayed as described in the legend to Figure 8 after preincubation at indicated concentrations of SCH 28080 and 2 μM ouabain in media containing 1 mM NaN_3 and 1 mM KCl (●) or 5 mM KCl (▲) or 25 mM KCl (■). The results of one out of three independent representative experiments which produced similar results are shown. Data are expressed as the percent of the maximal inhibition of the ATPase activity observed at 1 mM K^+. Curve fitting to the Michaelis−Menten equation was done using KaleidaGraph software.

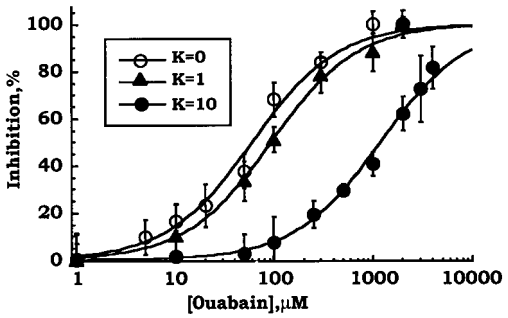

FIGURE 10: Potassium−ouabain antagonism. ATPase activity was determined after preincubation in the presence of the indicated concentrations of ouabain in assay medium containing 1 mM NaN_3 in the absence of added KCl (○) or in the presence of 1 mM KCl (▲) or 10 mM KCl (●). Data are expressed as the percent of the maximal inhibition of the ATPase activity observed in the absence of added K^+. Each value represents the mean ± SE of three or more independent representative experiments. Curve fitting to the Michaelis−Menten equation was done using KaleidaGraph software.

experiments we tested whether higher concentrations of K^+ would have an effect on SCH 28080 inhibition of ATP hydrolysis by the Atp1al1−βHK complex. As shown in Figure 9, sensitivity of the Atp1al1−βHK complex to SCH 28080 was already drastically reduced in the presence of 5 mM K^+ and further decreased at 25 mM K^+. Analysis of SCH 28080 inhibition was restricted to 1 mM concentration because a limited amount of inhibitor was available. Nevertheless, a rough estimation of apparent K_i values for SCH 28080 was made: 0.96 ± 0.14 mM at 5 mM K^+ and 1.56 ± 0.28 mM at 25 mM K^+. This increase in apparent K_i in the presence of increasing concentrations of K^+ is consistent with SCH 28080 competing with K^+ for activating ATPase activity.

The potassium−ouabain antagonism in the functioning of Na,K-ATPase is well established (*2*, *47*). Effects of increasing K^+ concentration on ouabain inhibition of ATP hydrolysis by the Atp1al1−βHK complex were analyzed in experiments of Figure 10. In these experiments, the specific Atp1al1−βHK ATPase activity was assayed at indicated concentrations of ouabain starting from 1 μM, the concentration which completely abolished the endogenous ouabain-sensitive ATPase activity presented in Sf-21 cell membranes but has practically no effect on the activity of the Atp1al1−βHK complex (Figure 3). It was shown that addition of K^+ protects the Atp1al1−βHK complex against inhibition by ouabain. The apparent K_i for ouabain increases from 60.4 ± 5.9 μM in nominally K^+-free medium to 95.8 ± 5.6 μM in the presence of 1 mM K^+ and to 1.20 ± 0.05 mM at 10 mM K^+. The apparent K_i for ouabain of 2.46 ± 0.3 mM was determined, when specific ATPase activity was measured in the presence of 50 mM K^+ and 100 mM Na^+ (data not shown). These observations clearly demonstrated competition between ouabain and potassium in the reaction of ATP hydrolysis by the Atp1al1−βHK complex.

DISCUSSION

The purpose of this study was to gain insight into enzymatic functions of the human nongastric H,K-ATPase, which until the discovery of the authentic β-subunit is represented by the recombinant Atp1al1−βHK complex. To achieve this goal, we have developed a procedure for the preparation of the functionally active Atp1al1−βHK complex in amounts sufficient for extensive biochemical studies using a baculovirus expression system.

Although substantial synthesis of both recombinant Atp1al1 and βHK proteins took place upon co-infection with the AcAtp1al1 and AcβHK viruses of both Sf-9 and Sf-21 cells, a significant level of formation of the functionally active Atp1al1−βHK complex was only detected in membranes of Sf-21 cells (Figure 2). Such a strong difference in efficiency of formation of the functional Atp1al1−βHK complex should not be considered as paradoxical, because according to previous observations, Sf-9 and Sf-21 cells may yield different levels and process recombinant proteins in slightly different ways (*32*). For example, expression of the SR Ca-ATPase in Sf-21 cells was described to be also much more efficient in comparison to that in Sf-9 cells (*48*).

Before proceeding to the functional characterization of the Atp1al1−βHK complex, it is necessary to consider the issue of endogenous ATPase activity of insect cells. An important advantage of the baculovirus expression system is that membranes of insect cells exhibit very little ouabain-sensitive ATPase activity. This was first revealed upon expression of different Na,K-ATPase isozymes in Sf-9 cells (*29*, *37*, *38*). The Sf-9 cell line is a clonal derivative of the Sf-21 cell line (*32*); therefore, it was not surprising that we identified the presence of small but detectable amounts of ouabain-sensitive activity in microsomes of noninfected Sf-21 cells. In respect to inhibitor sensitivity, this intrinsic ATPase activity is similar to that of the Na,K-ATPase (*2*): it is highly sensitive to ouabain, apparent K_i of 79 ± 12 nM (Figure 3), and completely resistant to SCH 28080 in concentrations up to 1 mM (data not shown). However, this ouabain-sensitive activity is most probably not a Na,K-ATPase, because Sf-21 cell membranes exhibit it in the absence of K^+ and Na^+ and its level does not change significantly in the presence of 1 mM K^+ and/or Na^+ (data not shown). These observations are consistent with previous studies (*37*), suggesting that the apparent ouabain-sensitive ATPase activity of insect cells is not a Na,K-ATPase.

Having determined that the Atp1al1 is effectively assembled with the βHK into the functionally active ATPase complex (Figure 2), we analyzed the ATPase activity with

respect to K^+, Na^+, pH, and ATP dependencies and inhibitor sensitivity. The accurate measurements of ATP hydrolysis by the Atp1al1−βHK complex were possible because endogenous ATPase activity of Sf-21 cell membranes was completely abolished in the presence of a few micromolar ouabain, the concentration which practically does not influence activity of the Atp1al1−βHK complex (Figure 3).

One of the most interesting observations made in this study was that the recombinant Atp1al1−βHK complex exhibits substantial specific ATPase activity in the absence of added K^+ in nominally K^+-free medium. This specific ATPase activity is moderately sensitive to both ouabain (Figure 3) and SCH 28080 (Figure 8). It is inhibited in a dose-dependent manner by ouabain with an apparent K_i of 64 ± 10 μM and by SCH 28080 with an apparent K_i of 94 ± 16 μM (Figure 8). With respect to ATPase activity in nominally K^+-free medium, properties of the recombinant Atp1al1−βHK complex are similar to that of the gastric H,K-ATPase, which in the absence of added K^+ exhibits significant specific ATPase activity (*39*, *42*, *45*). SCH 28080 inhibits this activity with a high affinity (apparent K_i is ∼30 nm), and inhibition is due to K^+-like prevention of phosphoenzyme formation (*45*). Although ATP hydrolysis by the Atp1al1−βHK complex in nominally K^+-free medium is much less sensitive to ouabain than Na,K-ATPase (*2*) and much less sensitive to SCH 28080 than gastric H,K-ATPase (*3*), the above observations indicated a certain similarity of spatial structure of membrane moieties of the Atp1al1, Na,K-ATPase, and gastric H,K-ATPase, as was suggested before (*4*, *12*). These structural aspects are discussed below.

It was previously reported that an increase in extracellular K^+ concentrations activates the Rb^+ uptake mediated by the Atp1al1−βHK complex expressed in *Xenopus* oocytes with a $K_{1/2}$ of ∼375 μM (*4*). As shown in Figures 4 and 5, ouabain-sensitive ATP hydrolysis by the Atp1al1−βHK complex was also stimulated by increasing concentrations of added K^+ in nominally Na^+-free medium in 1.5−3-fold (the apparent K_m value for added K^+ activation is 116 μM). This value of K_m is lower than that of the guinea pig nongastric H,K-ATPase expressed in HEK-293 cells, 0.68 mM (*14*). It is also lower than the K_m for K^+ activation of the Rb^+ uptake mediated by the Atp1al1−βHK complex expressed in HEK-293 cells, 1.92 mM (*17*). On the other hand, the K_m of the Atp1al1−βHK complex for ATP hydrolysis is very close to that for gastric H,K-ATPase that was determined to be within the 0.2−0.4 mM range for native as well as recombinant enzyme (*3*, *42*, *49*, *50*). K^+ activates ATP hydrolysis by Na,K-ATPase with K_m in the low millimolar range, but values are higher than that for the Atp1al1−βHK complex (see, for example, refs *2*, *29*, *37*, and *38*). However, this direct comparison is inappropriate, due to the need for Na^+ for the Na,K-ATPase activation (*41*).

An important goal of this study was to directly measure the effects of different Na^+ concentrations on Atp1al1−βHK ATPase activity, because previous experimental data regarding the influence of Na^+ on its functions were rather controversial and provided only indirect evidence. Thus, arguments in favor of Na^+ independence were that the same level of Rb^+ uptake mediated by the Atp1al1−βHK complex was detected in Na^+-loaded and unloaded *Xenopus* oocytes (*4*), something that contrasts with the strong activating effect of increasing intracellular Na^+ concentrations on Rb^+ uptake by Na,K-ATPase. A similar lack of the activating effect of Na^+ loading was also reported for the Atp1al1−βHK complex expressed in HEK-293 cells (*17*). On the other hand, a decrease in intracellular Na^+ concentration was detected in HEK-293 cells expressing the Atp1al1−βHK complex (*18*), and these cells were able to grow in the presence of 1 μM ouabain, which inhibits endogenous Na,K-ATPase (*18*). On the basis of these observations, it was suggested that the Atp1al1−βHK complex expressed in HEK-293 cells primarily mediates Na^+/K^+ rather than H^+/K^+ exchange (*18*). A significant decrease in intracellular Na^+ was also detected in *Xenopus* oocytes upon expression of the Atp1al1−βHK complex or Na,K-ATPase but not gastric H,K-ATPase, thus supporting the specificity of the effect observed with the Atp1al1−βHK complex (*30*; K. Geering et al., unpublished observations). Therefore, it was reasonable to expect that, similar to Na,K-ATPase, Na^+ may serve as an activator of ATP hydrolysis by the Atp1al1−βHK complex. However, the opposite effects were revealed in experiments in which ouabain-sensitive ATP hydrolysis was assayed in the presence of K^+ and Na^+ concentrations which varied from 0 to 50 mM and from 0 to 100 mM, respectively. As shown in Figure 5A, the Atp1al1−βHK complex exhibited the highest level of ATPase activity in the absence of added Na^+ in nominally Na^+-free medium, and an increase of Na^+ concentration in the assay medium resulted in a decrease of the ATP hydrolysis rate (Figure 5A). The inhibitory effect of Na^+ was detected at all K^+ concentrations tested (Figure 5B) but was the strongest in the absence of added K^+ (apparent K_i 24.3 ± 4.2 mM). Potassium partially protected the Atp1al1−βHK complex from Na^+ inhibition, and already in the presence of 1 mM K^+ the value of K_i increased about three times and remained at the same level (62−70 mM) up to 50 mM K^+. The close similarity of these K_i values may be explained by a high affinity of the Atp1al1−βHK complex for K^+ due to the fact that concentrations of K^+ higher than 1 mM should be considered as close to V_{max} conditions. Taken together, these results indicated that (a) Na^+ does not activate the ATP hydrolysis by the recombinant Atp1al1−βHK complex, but has an inhibitory effect on this reaction, and (b) there is an antagonism between K^+ stimulation and Na^+ inhibition in this reaction. Thus, with respect to the ATP hydrolysis reaction, the Atp1al1−βHK complex functions as a K^+-dependent ATPase that is inhibited by Na^+. It is well established that, in the case of Na,K-ATPase for which Na^+/K^+ exchange is an authentic function, the presence of Na^+ as an ion activator is an absolute requirement for manifestation of ATPase activity (for reviews, see refs *2*, *38*, and *41*). Thus, our results do not support the concept that a nongastric H,K-ATPase "is not an H,K-ATPase but rather acts as an Na,K-ATPase" (*18*). This contradictory issue remains to be explored further. At this point, it cannot also be excluded that, due to structural and functional similarity between all three groups of X,K-ATPases, the Atp1al1−βHK pump under particular conditions can accept replacement of protons by sodium ions and exhibit mixed specificity with respect to transporting cations.

Previous studies of ion sensitivity of other X,K-ATPases have demonstrated that Na^+ also partially inhibits ATP hydrolysis by the gastric H,K-ATPase but at an alkaline pH and has little or no effect on the activity at pH ≤7.0, thus revealing the possibility of competition between protons and

Na^+ on cytoplasmic sites (*3*, *51*). Sodium ions may also substitute for protons in the ion-transporting mechanism of the gastric H,K-ATPase at very low H^+ concentration (pH 8.5) but not at a neutral pH (*3*, *52*). In the case of Na,K-ATPase, K^+ has been shown to act as a competitive inhibitor of Na^+ binding at cytoplasmic sites, thus suggesting that Na^+/K^+ antagonism could be a plausible mechanism of pump regulation (for review, see ref *53*). Inhibitory effects of sodium ions on the kinetics of the ATP hydrolysis by the Atp1al1−βHK complex reported here suggested the existence of an intracellular Na^+ binding site on nongastric H,K-ATPase whose nature is hard to predict at the moment. Upon inhibition, Na^+ may directly affect the interaction of K^+ with the enzyme and/or may act through blockage of the reaction stages, which required proton participation. The answer cannot be predicted a priori, and this issue remains to be examined. It is very unlikely that Na^+ may strongly compete either with K^+ or with protons and inhibit ATPase reaction catalyzed by the nongastric H,K-ATPase under normal physiological conditions, since the concentration of K^+ in cells is about 10-fold higher than that of Na^+ and the K_i value for Na^+ inhibition is well above the intracellular Na^+ concentration. However, this does not exclude the possibility that this antagonism between intracellular cations might be involved in fine adjustment of the nongastric H,K-ATPase activity in vivo in response to changes in ion concentrations.

The data presented here have demonstrated that the ATP-hydrolyzing function of the Atp1al1−βHK complex is sensitive to both ouabain (Figures 3 and 10) and SCH 28080 (Figures 8 and 9). At 1 mM K^+, the apparent K_i values are practically the same for both inhibitors: 95.8 ± 5.6 μM for ouabain and 89 ± 11 μM for SCH 28080. These results are in accord with previous observations indicating that the ion-transporting function of the Atp1al1−βHK complex assayed by Rb^+ uptake in *Xenopus* oocytes (*4*) and HEK-293 cells (*17*) is moderately sensitive to ouabain and SCH 28080, with an apparent K_i of ∼13 μM (*4*) and ∼42 μM (*17*) for ouabain and ∼131 μM for SCH 28080 (*17*). Thus, with respect to sensitivity to these inhibitors, the Atp1al1−βHK complex (and presumably the authentic nongastric H,K-ATPase) occupies an intermediate position between Na,K-ATPase and gastric H,K-ATPase. The Na,K-ATPase isoforms (excluding rodent α1NK) have a K_i for ouabain in the nanomolar range and are resistant to SCH 28080 (*2*, *38*, *47*). In contrast, gastric H,K-ATPase is very sensitive to SCH 28080, with a K_i in the high nanomolar range, but is completely resistant to ouabain (*3*, *45*, *46*).

From the point of view of chemical structure and properties, ouabain and SCH 28080 are very different. However, the compounds exhibit common functional features: they inhibit Na,K-ATPase and gastric H,K-ATPase, respectively, through interaction with the extracytoplasmic surface of the target ion pumps, and both are strong competitors of potassium ions. Results of our experiments clearly demonstrated that antagonism between K^+ and either ouabain (Figure 10) or SCH 28080 (Figure 9) also exists in the reaction of ATP hydrolysis by the Atp1al1−βHK complex. Thus, the Atp1al1−βHK complex exhibits the highest sensitivity to ouabain in the absence of added K^+ with an apparent K_i of 60.4 ± 5.9 μM. Addition of 1 mM K^+ shifts the K_i to 95.8 ± 5.6 μM. The presence of 10 mM K^+ strongly protects the enzyme from inhibition, and the K_i is shifted into the low millimolar range (1.20 ± 0.05 mM) (Figure 10). In the case of SCH 28080 inhibition of the Atp1al1−βHK complex, the presence of K^+ in concentrations up to 1 mM does not have an effect on inhibitor−enzyme interaction, and apparent K_i values in nominally K^+-free medium and at 1 mM K^+ are practically the same (94 ± 16 μM and 89 ± 11 μM) (Figure 8). However, further increase in K^+ concentration drastically reduces sensitivity to SCH 28080, and the apparent K_i is shifted to the millimolar range (0.96 ± 0.14 mM at 5 mM K^+ and 1.56 ± 0.28 mM at 25 mM K^+) (Figure 10). Studies of ouabain and SCH 28080 effects on other partial reactions of nongastric H,K-ATPase are needed to define which concrete step in functioning is affected by each inhibitor.

As it was mentioned above, ouabain and SCH 28080 molecules are very different in size and shape, suggesting different construction of their binding sites formed by extracellular and transmembrane domains of the α-subunits of Na,K-ATPase and gastric H,K-ATPase, respectively. However, inhibition of the Atp1al1−βHK complex by both ouabain and SCH28080 indicates that certain essential elements of binding sites to both inhibitors coexist in the Atp1al1 polypeptide chain. Comparison of sequences of the Atp1al1 and the Na,K-ATPase α-subunit reveals that the majority of the amino acid residues that have been identified by in vitro mutagenesis as important for ouabain sensitivity of the Na,K-ATPase (summarized in ref *54*) are also present at the corresponding positions in the Atp1al1, e.g., Cys 126, Tyr 130, Gln 133, Asn 144, Tyr 330, Leu 815, Thr 819, Tyr 885, Arg-902, and Phe 1005 (*12*). In contrast, the extracellular loop between the first and second transmembrane segments of Na,K-ATPase, which is thought to be an essential part of the digitalis receptor and Atp1al1, shares only 2 out of 12 residues. This stretch of high-sequence divergence may explain the lower ouabain sensitivity of nongastric H,K-ATPase.

It was recently shown by mutagenic alterations of the gastric H,K-ATPase that Cys 321, Cys 813, Ile 816, Glu 820, Thr 823, and Pro 827 from the segments TM3 and TM6 are involved in determining the affinity for SCH 28080 (*55*−*57*). Three of these residues (Ile, Thr, and Pro) are conserved in all X,K-ATPases. Cys 813 is substituted in Atp1al1 and other X,K-ATPases by Thr, which is involved in determination of Na,K-ATPase sensitivity to ouabain (*54*). It is interesting to note that the position of Glu 820 is occupied by Asp in Atp1al1, and mutation Glu820Asp in the gastric pump resulted in 8 times lower SCH 28080 sensitivity (*55*). This value is still much higher than that of the Atp1al1−βHK complex. This indicates that determinants of SCH 28080 sensitivity are not limited to the above amino acid residues.

According to our previous studies (*16*, *22*, *24*), the authentic counterpart of the Atp1al1 in vivo is, most probably, not the β-subunit of gastric H,K-ATPase but rather a hitherto unidentified β-subunit with structural features resembling that of βHK. In case of Na,K-ATPases, it has been shown that the nature of the β-subunit influences the cation sensitivity and the enzymes formed by the α-subunit assembled with different β-isoforms exhibited detectable differences in apparent affinities for K^+ and Na^+ (*38*, *58*). Therefore, at this point we cannot exclude the possibility that real ion sensitivity of the human nongastric H,K-ATPase

(i.e., Atp1al1 combined with authentic β-subunit) might be different (in terms of the actual values) from that of the Atp1al1−βHK complex. The undoubted identification of the real β-subunit of nongastric H,K-ATPase is needed to clarify this issue.

In conclusion, we have developed a procedure for the preparation of the human nongastric H,K-ATPase represented by the recombinant Atp1al1−βHK complex expressed in Sf-21 insect cells in amounts sufficient for extensive biochemical studies. These first direct measurements of the catalytic functions with respect to the ATP hydrolysis reaction clearly demonstrate that the recombinant complex exhibits enzymatic properties of K^+-dependent ATPase sensitive to ouabain, SCH 28080, and Na^+. Its pH, ATP dependencies, and sensitivity to vanadate are within the range of values that are common for all known X,K-ATPases. It differs from Na,K-ATPase in cation dependence and differs from gastric H,K-ATPase and Na,K-ATPase in sensitivity to inhibitors. We anticipate that further studies of the functional characteristics of native and mutant forms of the nongastric H,K-ATPase, including reactions of phosphorylation from ATP or inorganic phosphate and dephosphorylation and analysis of phosphatase activity, which are in progress, will provide essential information for the understanding of this X,K-ATPase reaction mechanism.

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