Functional Expression of the Colonic H\(^{+}\),K\(^{+}\)-ATPase \(\alpha\)-Subunit

PHARMACOLOGIC PROPERTIES AND ASSEMBLY WITH X\(^{+}\),K\(^{+}\)-ATPase \(\beta\)-SUBUNIT\(\text{\textsuperscript{*}}\)

(Received for publication, August 6, 1996)

Juan Codina, Bruce C. Kone‡, Juan T. Delmas-Mata, and Thomas D. DuBose, Jr.§

From the Departments of Internal Medicine and Integrative Biology, The University of Texas Medical School at Houston, Houston, Texas 77030

The functional and pharmacological properties of the \(\alpha\)-subunit of the colonic H\(^{+}\),K\(^{+}\)-ATPase \(\alpha_{C}\) were studied in Xenopus laevis oocytes. \(\alpha_{C}\) was injected with different rat \(\beta\)-subunits, the \(\beta\)-subunit of the gastric H\(^{+}\),K\(^{+}\)-ATPase \(\beta_{0}\) (the only H\(^{+}\),K\(^{+}\)-ATPase \(\beta\)-subunit identified in rat), or the \(\beta\)-subunit of the Na\(^{+}\),K\(^{+}\)-ATPase \(\beta_{1}\) (associated with the basolateral Na\(^{+}\),K\(^{+}\)-ATPase, but also expressed in the epithelial apical membranes of rat distal colon) (Marxer, A., Stieger, B., Quarini, A., Kugler, M., and Hauri, H. P. (1989) J. Cell Biol. 109, 1057–1069). The effect of the different \(\beta\)-subunits was studied by measuring \(^{86}\text{Rb}\)\(^{+}\) uptake (a K\(^{+}\) congener) in the presence or absence of Sch-28080 and ouabain. Significant Na\(^{+}\)-independent \(^{86}\text{Rb}\)\(^{+}\) uptake was observed only when \(\alpha_{C}\) was coexpressed with one of the \(\beta\)-subunits. The expressed \(\alpha_{C}\beta_{1}\) and \(\alpha_{C}\beta_{0}\) complexes were not inhibited by Sch-28080, were only partially sensitive to ouabain (IC\(_{50}\) = 400–600 \(\mu\text{M}\), in the presence of external 1 \(\mu\text{M}\) KCl), and exhibited comparable K\(^{+}\) activation kinetics. Coexpression of \(\alpha_{C}\) with epitope-tagged \(\beta_{0}\) or \(\beta_{1}\), followed by immunopurification of the \(\alpha\beta\) complexes, confirmed stable assembly of \(\alpha_{C}\beta_{1}\) and \(\alpha_{C}\beta_{0}\) complexes. Since the \(\beta_{1}\)-subunit, but not the \(\alpha\)-subunit, of Na\(^{+}\),K\(^{+}\)-ATPase is expressed in the apical membrane of rat colonocytes, our data support the view that, in rat distal colon, the \(\beta_{1}\)-subunit may play a surrogate role as the \(\beta\)-subunit for the colonic H\(^{+}\),K\(^{+}\)-ATPase.

The H\(^{+}\),K\(^{+}\)-ATPase comprises a group of integral membrane proteins that belong to the X\(^{+}\),K\(^{+}\)-ATPase\(\text{\textsuperscript{\textsuperscript{1}}}\) subfamily of P-type cation-transporting ATPases (1). The X\(^{+}\),K\(^{+}\)-ATPases, which also include the Na\(^{+}\),K\(^{+}\)-ATPase isozymes, share a common catalytic cycle, the ability to extrude a cation (Na\(^{+}\) or H\(^{+}\), respectively) from the cell in exchange for K\(^{+}\), and an apparent requirement for heterodimeric structure (2–4). To date, cDNAs encoding homologous H\(^{+}\),K\(^{+}\)-ATPase \(\alpha\)-subunits have been cloned from gastric parietal cells (\(\alpha_{G}\) or HKa1) (5, 6), rat and guinea pig distal colon (\(\alpha_{Cl}\) or HKa2) (7, 8), toad urinary bladder (\(\alpha_{Bl}\) or HKa3) (9), and human skin (ATP1AL1 or HKa4) (10). Although these H\(^{+}\),K\(^{+}\)-ATPase isoforms share approximately 60–70\% amino acid identity, they exhibit distinct kinetic and pharmacological properties when expressed in heterologous systems (9, 11, 12). While the physiological role of the gastric H\(^{+}\),K\(^{+}\)-ATPase isoform in mediating gastric acid secretion is widely recognized, the biological roles of the other isoforms have not been clearly established.

Recent molecular biological and biochemical studies indicated that the colonic H\(^{+}\),K\(^{+}\)-ATPase participates in the chronic adaptation to changes in K\(^{+}\) homeostasis (7). While K\(^{+}\) balance is governed principally by the kidney, the colon plays a smaller but highly significant role. During chronic K\(^{+}\) restriction, active K\(^{+}\) reabsorption by epithelial cells of the renal collecting duct and distal colon serves to restore K\(^{+}\) balance (13). K\(^{+}\)-ATPase activities are expressed in these cell types, and these activities are up-regulated during chronic dietary K\(^{+}\) depletion. The K\(^{+}\)-ATPase activity in the renal collecting duct was reported to be ouabain-resistant and Sch-28080-sensitive, findings compatible with the established properties of \(\alpha_{Cl}\) (14). However, the fact that expression of \(\alpha_{Cl}\) mRNA is not significantly altered in the medullary collecting duct during chronic dietary K\(^{+}\) depletion (15), and that it is not expressed in the distal colon, makes it unlikely that this gene plays a role in K\(^{+}\) adaptation in either the kidney or the distal colon (16). In contrast, \(\alpha_{Cl}\) mRNA is principally expressed in the renal collecting duct and surface epithelial cells of the distal colon, and its abundance increases 3–5-fold in the renal medulla during chronic dietary K\(^{+}\) depletion (17). Thus, \(\alpha_{Cl}\) has emerged as the candidate gene most likely to mediate K\(^{+}\) conservation. Consequently, considerable effort has been devoted to define the functional properties of \(\alpha_{Cl}\).

Biochemical studies found that the apical membranes of surface epithelial cells of distal colon express distinct ouabain-sensitive (18, 19) and ouabain-insensitive (19, 20) K\(^{+}\)-ATPase activities. Since \(\alpha_{Cl}\) is the only X\(^{+}\),K\(^{+}\)-ATPase \(\alpha\)-subunit known to be expressed in the apical membranes of these cells, these results suggested either the existence of a novel \(\alpha\)-subunit, or the possibility that different \(\alpha\)-\(\beta\)-complexes exhibit different ouabain sensitivities. The latter possibility gained credence with the recent conflicting reports regarding the pharmacological properties of \(\alpha_{Cl}\) expressed in heterologous systems. Lee et al. (11) demonstrated that expression of \(\alpha_{Cl}\) without an exogenous \(\beta\)-subunit in baculovirus-infected Spodoptera frugiperda (SF-9) cells yielded K\(^{+}\)-ATPase activity that was resistant to high concentrations (1 mM) of ouabain, but inhibited by high concentrations (100 \(\mu\text{M}\)) of Sch-28080. Subsequently, Cougnon et al. (12) reported that coinjection of Xenopus laevis electrophoresis; mAb, monoclonal antibody; X\(^{+}\), undesignated cation.

\(\text{\textsuperscript{\textsuperscript{*}}}\) This work was supported in part by National Institutes of Health Grants DK-30603 (to T. D. D.) and DK-47981 (to B. C. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(\text{\textsuperscript{‡}}\) An Established Investigator of the American Heart Association during this work.

\(\text{\textsuperscript{§}}\) To whom correspondence should be addressed: Dept. of Internal Medicine, Division of Renal Diseases and Hypertension, The University of Texas Medical School at Houston, Houston, TX 77030. Tel.: 713-792-5425; Fax: 713-794-1197; E-mail: tdubose@heart.med.uth.tmc.edu.

\(\text{\textsuperscript{1}}\) The abbreviations use are: \(\alpha_{G}\), \(\beta_{1}\)-subunit of the gastric H\(^{+}\),K\(^{+}\)-ATPase (also called HKa1); \(\alpha_{Cl}\), \(\beta_{0}\)-subunit of the colonic H\(^{+}\),K\(^{+}\)-ATPase; \(\beta_{1}\), \(\beta_{0}\)-subunit of the Na\(^{+}\),K\(^{+}\)-ATPase; NMDG, N-methyl-D-glucosamine; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; X\(^{+}\), undesignated cation.
oocytes with cRNAs encoding αC and a β-subunit from toad bladder (β3) resulted in functional H+,K+-ATPase activity that was described as ouabain-sensitive (IC50 ~ 970 μM at 5 mM external K+ concentration) but Sch-28080-resistant. Neither study, however, examined the expression of an H+,K+-ATPase holoenzyme comprised of αC and a mammalian β-subunit, nor did they directly establish whether αC can complex with any of the known β-subunits. These limitations assume considerable importance when one considers that different β-subunits, when coexpressed with the α-subunits of the gastric H+,K+-ATPase or the Na+,K+-ATPase, may confer unique functional properties on the holoenzyme (21, 22).

Accordingly, we used the oocyte expression system to examine the functional properties of αC, when it is coexpressed with either of two rat X+,K+-ATPase β-subunits: the β-subunit of the gastric H+,K+-ATPase (βa), which is expressed in renal collecting duct (24) but not distal colon (23), and the βb-subunit of the Na+,K+-ATPase (βb), which is expressed in both collecting duct (24) and in apical and basolateral membranes of colonocytes (25) in distal colon. We used this expression system instead of the baculovirus system, because SF9 cells exhibit endogenous H+/H+ exchange (26) that would likely confound interpretation of K+ activation kinetics for a heterologously expressed H+,K+-ATPase. In addition, we constructed βbC- and βb-subunits bearing a common c-myc epitope to test, in a coimmunoprecipitation assay (27), whether αC stably assembles with βb and/or βb. The results indicate that αC can interact with both β-subunits, that oligomerization is required for functional activity of the expressed enzymes in this system, and that the inhibitor sensitivities and K+ activation kinetics of αCβb and αCβb holoenzymes are very similar. The ability of βb to support functional activity of αC, combined with the colocalization of these subunits in the apical membrane of the colonocytes (25) suggests that αCβb likely represents at least one of the K+-ATPase activities expressed in this locale, and that the existence of a “βb”-subunit need not be necessarily invoked.

EXPERIMENTAL PROCEDURES

Materials—The αC, (7) and αC (15) cDNAs were gifts from Dr. G. Shull (University of Cincinnati). The βb (25) was a gift from Dr. T. A. Pressey (Texas Tech University). The βb (25) cDNA was cloned as described below. The expression vector pAGA#2 was a gift from Dr. L. Birnbaum (University of California at Los Angeles) (26). mAb 9E10 was purified from culture supernatants of hybridoma myc 1–9E10.2 (American Type Culture Collection, Rockville, MD). Restriction enzymes were from Promega Biotech Inc. and New England Biolabs (Beverly, MA). T7 Cap Scribe was from Boehringer Mannheim. The X. laevis oocytes were prepared and injected in Dr. L. Parent’s laboratory (Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX). Oligonucleotides were synthesized by Genosys (The Woodlands, TX). The remaining reagents were of the best available quality.

Cloning of cDNA Encoding the Gastric H+,K+-ATPase β-Subunit—The complete open reading frame of βa was generated by a polymerase chain reaction using cDNA prepared from total rat stomach RNA and primers based on the published sequence (23), according to the conditions previously described by our laboratory (16). The sense oligonucleotide (5′-ATTCCTGCGACCTGCGAGGAG-3′) contained an Ncol site (underlined), and the antisense oligonucleotide (5′-TACG-GTTCAGTTCTCACTTGGTT-3′) contained a SalI site (underlined) to facilitate subcloning into pAGA#2. The correct sequence was verified by direct sequencing (30) of one of the clones.

cRNA Synthesis and Protein Expression in Xenopus Oocytes—Encoding DNAs for rat αC, αC, βa, βb, and βb-subunits were subcloned into pAGA#2. The recombinant molecules were linearized with HindIII or XhoI as appropriate, and capped RNAs were synthesized using T7 RNA polymerase and T7 Cap Scribe (Boehringer Mannheim) according to the manufacturer’s methods. Parametric studies demonstrated that full-length cRNAs were generated for each gene. Stage V–VI oocytes obtained from X. laevis were injected with 10 ng of cRNA or an equivalent volume of water and incubated at 19°C in modified Barth’s medium (31). Three days later, 36Rb uptake was measured according to the procedure described by Modyanov et al. (1). Briefly, the oocytes were incubated with 180 mM NaCl (in the presence 90 mM NMDG was used), 1 mM MgCl2, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, 5 mM BaCl2, and 10 mM PIPES, pH 7.4 for 15 min. Subsequently oocytes were preincubated in the presence or absence of inhibitors (ouabain or Sch-28080) in buffer A for 15 min. The oocytes were then incubated in buffer A containing 5·10–10 cm 36Rb– and 1–5 mM KCl in the presence or absence of inhibitors. All incubations were performed at room temperature, and 1 ml of each incubation was added as described to inhibit the activity of endogenous X. laevis Na+ + K+-ATPase (1). After 15 min, the reaction was stopped by aspirating the bulk of the radioactivity and washing three times with 4 ml of buffer A at 4°C. Finally, the oocytes were disrupted by pipetting up and down, transferred to scintillation vials, and counted. The quantities of 36Rb uptake were then calculated. The (no oocyte) of the experiment were routinely less than 200 cpm.

Subunit Assembly Assay—A human c-myc epitope tag, recognized by human-specific mAb 9E10 (32), was fused to the amino terminus of βa and βb by inserting a double-stranded oligonucleotide adapter (sense 5′-CATGGACGAAAAGCTGATCTCCGAGGAGGACCT-3′; antisense, 5′-CATGAGGTCCTCCTCGGAGATCAGCTTTTGCTC-3′) that contained an initiation ATG followed by nucleotides encoding the c-myc-epitope (EQKLISEEDL), into each β-subunit. The resultant recombinant molecules were termed c-myc-βa and c-myc-βb. The addition of the correct nucleotide sequence was confirmed by DNA sequencing. Oocyte proteins were metabolically labeled by coinjection of 0.345 μCi [35S]methionine with the α and c-myc-β-subunit cRNAs. Three days after the injection, the oocytes were extracted and the proteins were extracted by incubating for 50 min at 4°C in the presence of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% Triton X-100 (buffer B) (27). The insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4°C. The extracted proteins from 5–10 oocytes were pooled and incubated with 2 μg of mAb 9E10 for 4 h at 4°C, followed by addition of 10 μl (packed volume) of protein A/G plus agarose (Santa Cruz Biotechnology). After 2 h of vigorous shaking, the resin was washed six times with buffer B. The bound protein was extracted from the resin with Laemmli sample buffer containing 10% β-mercaptoethanol and separated on SDS-10% polyacrylamide gels. The gels were then fixed in 7% glacial acetic acid, 25% methanol and impregnated with 2,5-diphenyl-tetrazolium chloride. After 3 days, the gels were dried and exposed to Kodak XAR-5 film with intensifying screens at –70°C for 1–3 days. The coprecipitation of an α-subunit with the c-myc-βa, or c-myc-βb-subunit using this protocol was interpreted as subunit assembly in accordance with previous studies of Na+,K+-ATPase αβ assembly (27).

Data Analysis—Quantitative data are presented as means ± S.E. and were tested for significance by analysis of variance. p < 0.05 was taken as significant.

RESULTS

Functional Properties of αCβa or αCβb Expressed in Xenopus Oocytes—Sets of oocytes were injected with cRNAs for αC, αC, βa, βb, αC plus βa, or αC plus βb. The controls were injected with water alone. 36Rb uptake was measured 3 days later in the presence of 5 mM KCl. When the oocytes were injected with one subunit only, there was no effect on 36Rb uptake compared to water-injected controls (Fig. 1). However, when oocytes were injected with αC plus βa or αC plus βb there was a significant increase in 36Rb uptake compared to any group injected with one subunit alone or the control group. This 36Rb uptake was independent of the presence of Na+ in the incubation medium; equimolar replacement of external Na+ by NMDG did not alter the 36Rb uptake in any group. This result established that the 36Rb uptake was not mediated by the Na+,K+-ATPase.

To assess the K+ activation kinetics of the αCβa or αCβb complexes, 36Rb uptake was assayed in αC plus βa, or αC plus βb-expressing oocytes in the presence of increasing concentrations of KCl in the uptake buffer (Fig. 2). External K+ activated 36Rb uptake in these oocytes in a concentration-dependent, saturable manner. Lineweaver-Burk transformation of the kinetic data revealed K1/2 values for K+ activation of 1.4 mM when the oocytes expressed the αCβa complex and 1.8 mM when the αCβb complex was expressed. Both values are slightly higher than the value (1.2 mM) reported for SF-9 cells express-
Coexpression of $\alpha_C$ with $\beta_G$ or $\beta_1$

FIG. 1. $^{86}\text{Rb}^+$ uptake in oocytes expressing $\alpha_1\beta_0$, or $\alpha_2\beta_1$, complexes. Oocytes were injected with cRNAs encoding $\beta_0$, $\alpha_1$, $\alpha_2$, or $\alpha_C$, $\alpha_1$ plus $\beta_0$, or $\alpha_C$ plus $\beta_1$, or water alone. Three days later, $^{86}\text{Rb}^+$ uptake was measured in the presence of 5 mM KCl and either 90 mM NaCl (open bars) or 90 mM NMDG (closed bars). Data are means of 10–12 oocytes.

FIG. 2. K⁺-dependent activation of $^{86}\text{Rb}^+$ uptake in oocytes coexpressing $\alpha_C$ and $\beta_C$, or $\beta_1$-subunit. Top panel, oocytes were coinjected with $\alpha_C$ and $\beta_C$ (diamonds) or with $\alpha_C$ and $\beta_1$ (circles). Three days later, $^{86}\text{Rb}^+$ uptake was measured in the presence of 90 mM NaCl at the indicated external KCl concentrations. Bottom panel, the Lineweaver-Burk plot of the same data. Each point on the plot represents the data obtained from 8–10 oocytes. Straight lines were fitted by method of least squares. Correlation coefficients ($R$) were 0.9 for both $\alpha_C\beta_C$ and $\alpha_C\beta_1$.

FIG. 3. Effect of ouabain concentration on $^{86}\text{Rb}^+$ uptake in oocytes expressing $\alpha_1\beta_0$, or $\alpha_2\beta_1$, complexes. $^{86}\text{Rb}^+$ uptake was determined in the presence of 1 mM KCl and either 90 mM NaCl or 90 mM NMDG (closed bars). Data are means of 10–12 oocytes. **$p < 0.01$ versus no ouabain. Open bars, no inhibitor; closed bars, incubated in presence of Sch-28080. Ten oocytes were used in each group.

FIG. 4. Effect of Sch-28080 concentration on $^{86}\text{Rb}^+$ uptake in oocytes expressing $\alpha_C\beta_G$, $\alpha_C\beta_1$, or $\alpha_C\beta_3$ complexes. $^{86}\text{Rb}^+$ uptake, in oocytes coexpressing $\alpha_C$ and $\beta_G$, $\alpha_C$ and $\beta_1$, $\alpha_C$ and $\beta_3$, or $\alpha_C$ plus the $\beta_i$ subunits, was determined in the presence of 1 mM KCl, 90 mM NaCl and the indicated concentrations of Sch-28080. **$p < 0.01$ versus no Sch-28080. Open bars, no inhibitor; closed bars, incubated in presence of Sch-28080. Ten oocytes were used in each group.

The $^{86}\text{Rb}^+$ uptake experiments indicated that $\alpha_C$ must be coexpressed with $\beta_0$ and $\beta_1$ for holoenzyme function, we sought to establish directly that stable $\alpha_C\beta_0$ and $\alpha_C\beta_1$ complexes were formed. The human c-myc epitope was added to the amino terminus of $\beta_3$ (c-myc-$\beta_0$) and of $\beta_1$ (c-myc-$\beta_1$) (27). Oocytes were injected with $\alpha_C$, c-myc-$\beta_0$, c-myc-$\beta_1$, $\alpha_C$ plus c-myc-$\beta_0$, or $\alpha_C$ plus c-myc-$\beta_1$. The Triton X-100 extracts were immunoprecipitated with mAb 9E10 (27), and samples were analyzed by SDS-PAGE and fluorography. Fig. 5 demonstrates that mAb 9E10 did not immunoprecipitate $\alpha_C$ when it was expressed alone (without a $\beta_i$-subunit). However, when $\alpha_C$ cRNA was coinjected with c-myc-$\beta_0$, cRNA, the two subunits were coprecipitated, indicating stable assembly. As predicted from the $^{86}\text{Rb}^+$ uptake data, stable assembly between $\alpha_C$ and c-myc-$\beta_1$ was also evident when the oocytes were coinjected with cRNAs for these subunits (Fig. 5).

The relative amounts of $\alpha_C\beta_0$ and $\alpha_C\beta_3$ coprecipitated in this assay were roughly comparable. The immunoprecipitated $\beta_0$ and $\beta_1$ were in the core glycosylated (narrow band at ~50 kDa) and fully glycosylated (broad band at 60–70 kDa) forms. Both the core and fully glycosylated $\beta_i$-subunits migrated more rapidly than the corresponding glycosylated forms of $\beta_3$ on SDS-PAGE, as observed by others (34). The explanation for such differences in mobility is unknown. A band that migrated near $\alpha_C$ (labeled ? in Fig. 5) was also immunoprecipitated from oocytes injected with cRNA for $\beta_0$ or $\beta_1$, regardless of whether $\alpha_C$ cRNA was coinjected. Presumably this band represents the endogenous $\alpha_i$-subunit of the Na⁺,K⁺-ATPase.

Pharmacological Properties of $\alpha_C$ Coexpressed with Different $\beta$-Subunits—In the presence of 1 mM external K⁺, ouabain inhibited $^{86}\text{Rb}^+$ uptake of oocytes expressing $\alpha_C\beta_0$ or $\alpha_C\beta_1$ in a dose-dependent manner, with IC₅₀ values of ~390 and ~640 µM, respectively (Fig. 3). In contrast, Sch-28080, at concentrations up to 500 µM, failed to inhibit $^{86}\text{Rb}^+$ uptake of oocytes coexpressing $\alpha_C\beta_0$ or $\alpha_C\beta_1$ (Fig. 4). In positive control experiments, 10 µM Sch-28080 abolished $^{86}\text{Rb}^+$ uptake of oocytes coexpressed with $\alpha_C$ plus $\beta_0$ cRNAs, in agreement with previous reports (33).

Assembly of $\alpha_C\beta_0$ and $\alpha_C\beta_1$ Complexes—Since the $^{86}\text{Rb}^+$ uptake experiments indicated that $\alpha_C$ was coexpressed with $\beta_0$ and $\beta_1$ for holoenzyme function, we sought to establish directly that stable $\alpha_C\beta_0$ and $\alpha_C\beta_1$ complexes were formed. The human c-myc epitope was added to the amino terminus of $\beta_3$ (c-myc-$\beta_0$) and of $\beta_1$ (c-myc-$\beta_1$) (27). Oocytes were injected with $\alpha_C$, c-myc-$\beta_0$, c-myc-$\beta_1$, $\alpha_C$ plus c-myc-$\beta_0$, or $\alpha_C$ plus c-myc-$\beta_1$. The Triton X-100 extracts were immunoprecipitated with mAb 9E10 (27), and samples were analyzed by SDS-PAGE and fluorography. Fig. 5 demonstrates that mAb 9E10 did not immunoprecipitate $\alpha_C$ when it was expressed alone (without a $\beta_i$-subunit). However, when $\alpha_C$ cRNA was coinjected with c-myc-$\beta_0$, cRNA, the two subunits were coprecipitated, indicating stable assembly. As predicted from the $^{86}\text{Rb}^+$ uptake data, stable assembly between $\alpha_C$ and c-myc-$\beta_1$ was also evident when the oocytes were coinjected with cRNAs for these subunits (Fig. 5).

The relative amounts of $\alpha_C\beta_0$ and $\alpha_C\beta_3$ coprecipitated in this assay were roughly comparable. The immunoprecipitated $\beta_0$ and $\beta_1$ were in the core glycosylated (narrow band at ~50 kDa) and fully glycosylated (broad band at 60–70 kDa) forms. Both the core and fully glycosylated $\beta_i$-subunits migrated more rapidly than the corresponding glycosylated forms of $\beta_3$ on SDS-PAGE, as observed by others (34). The explanation for such differences in mobility is unknown. A band that migrated near $\alpha_C$ (labeled ? in Fig. 5) was also immunoprecipitated from oocytes injected with cRNA for $\beta_0$ or $\beta_1$, regardless of whether $\alpha_C$ cRNA was coinjected. Presumably this band represents the endogenous $\alpha_i$-subunit of the Na⁺,K⁺-ATPase.
The functional and pharmacologic properties of this isoform, and its requirements for association with a β-subunit remain ambiguous. We used the oocyte expression system to study the properties of αC, interacting with differing β-subunits. The rat β3-subunit was selected for study because it is the only known mammalian H\(^+\)K\(^-\)ATPase β-subunit, and it is coexpressed with αC in the renal collecting duct (4, 38). The rat Na\(^+\)K\(^-\)ATPase β1-subunit was chosen because immunoreactivity for this protein was found in the apical membrane of rat colonocytes (25), a membrane domain in which Na\(^+\)-independent K\(^-\)ATPase activity, but not Na\(^+\),K\(^-\)ATPase activity or α2-subunit immunoreactivity, was observed. We therefore hypothesized that both β1 and β3 would support αC functional activity.

In agreement with the majority of studies of X\(^+\),K\(^-\)ATPases (9, 12, 23), our \(^{86}\)Rb\(^+\) uptake data provide clear evidence that αC requires a β-subunit for functional activity in the oocyte expression system. Cougnon et al. (12), using αC coexpressed in oocytes with an amphibian β-subunit, reached a similar conclusion. As predicted from our \(^{86}\)Rb\(^+\) uptake data, αC was coprecipitated with either the βC or β3-subunit in the assembly assay, confirming the formation of stable heterodimers. The fact that comparable amounts of αCβC and αCβ3 complexes were coprecipitated in the assembly assay (Fig. 5) suggests that the assembly efficiency and/or stability of these heterodimers are quite similar. Using an identical assembly assay, Lemas et al. (27) found that the Na\(^+\),K\(^-\)ATPase α2-subunit can assemble with β1 or β3, and they identified a 26 amino acid region of the Na\(^+\),K\(^-\)ATPase α2 sufficient to allow stable interaction with these β-subunits. In addition, Jaisser et al. (22) found that β1 could support functional activity of αC. Our data, therefore, extend the range of potential αβ pairs to include αCβ3 and αCβ1, and lend further support to the concepts that X\(^+\),K\(^-\) ATPase α-subunits contain a conserved assembly domain for β-subunit association, and that there is no remarkable αβ isofrom selectivity in the assembly process.

Previous studies suggested that the different β-subunit isoforms could confer different K\(^-\)-activation kinetics on the Na\(^+\),K\(^-\)ATPase. Coexpression of the Bufo Na\(^+\),K\(^-\)ATPase α1-subunit with the Bufo Na\(^+\),K\(^-\)ATPase β1-subunit, Na\(^+\),K\(^-\)ATPase β3-subunit, or rabbit H\(^+\),K\(^-\)ATPase β3-subunit in Xenopus oocytes resulted in different K\(^-\)-activation kinetics for the various holoenzymes. The Na\(^+\),K\(^-\)ATPase α1(H\(^+\)),K\(^-\)ATPase β3 enzyme performed as a Na\(^+\),K\(^-\) pump with a much lower apparent affinity for K\(^-\), both in the presence and absence of external Na\(^+\), compared to the Na\(^+\),K\(^-\)ATPase α1/Na\(^+\),K\(^-\)ATPase β1 and Na\(^+\),K\(^-\)ATPase α1/Na\(^+\),K\(^-\)ATPase β3 pumps (39). In the present report, however, αCβ1 and αCβ3 enzymes exhibited comparable K\(^-\)-activation kinetics. Thus the ability of different β-subunits to alter this functional property may be restricted to specific X\(^+\),K\(^-\)ATPase α-subunits or to hybrid ion pumps of specific species.

Structure-function studies of the Na\(^+\),K\(^-\)ATPase α-subunit indicated that amino acids in several transmembrane regions (H1, H2, H5, and H6), the first extracellular loop, and the third extracellular loop (specifically Cys\(^{121}\), Tyr\(^{124}\), Glu\(^{137}\), Asn\(^{123}\), and Tyr\(^{311}\), Phe\(^{316}\), Leu\(^{318}\), Thr\(^{319}\), Phe\(^{321}\), Arg\(^{323}\)), contributed to ouabain sensitivity (40, 41). Comparison of the amino acid composition of αC with the Na\(^+\),K\(^-\)ATPase α-subunits indicates that most of these amino acids are conserved, with the notable exception that Tyr\(^{311}\) and Phe\(^{316}\) of the Na\(^+\),K\(^-\)ATPase α-subunit are substituted with Phe and Tyr in αC. The specific role of these two amino acids in conferring relative ouabain-insensitivity to αC has not yet been studied. It has also been suggested that Phe\(^{314}\) and Asp\(^{137}\) (rat αC sequence) (6) are required to confer Sch-28080-sensitivity to αC, but the αC and α2 sequences are identical at these two positions. Given evidence that αC is Sch-28080-insensitive (12) (present report), sequences other than these two amino acids must be implicated in Sch-28080 binding affinity to the α-subunit. One candidate motif for conferring Sch-28080 sensitivity is the sequence GDLT (amino acids 131–134 of αC), which is conserved in the known αC(α2)-subunits (all of which are Sch-28080-sensitive) of amphibians, birds and mammals, but is absent from αC (6). Pharmacologic analysis of chimeric αC/α2 molecules and site-directed mutants of αC should clarify this question.

Functional studies have suggested the presence of both ouabain-sensitive and -insensitive components of K\(^-\)-ATPase activity in rat distal colon (11, 18, 19, 20, 42) and kidney (43). In addition, a polyclonal antibody directed against the amino terminus of αC inhibited both ouabain-sensitive and -insensitive components of K\(^-\)-ATPase activity in apical membranes prepared from distal colon, and it specifically labeled only the apical membrane of the distal colon epithelium (11). Since this antibody did not label the basolateral membrane, where the Na\(^+\),K\(^-\)ATPase resides in these cells, and did not inhibit Na\(^+\),K\(^-\)ATPase activity in membranes from rabbit renal medulla, this antibody does not appear to cross-react with the Na\(^+\),K\(^-\)ATPase α1-subunit. Moreover, whereas 1 mM ouabain failed to inhibit K\(^-\)-ATPase activity in αC-expressing SF-9 cells, it dramatically inhibited by 75% K\(^-\)-ATPase activity of colonic apical membranes. Integration of these data with the present results suggests that the ideal that, in the apical membrane of the distal colon epithelium, αCβ1 holoenzymes contribute the Sch-28080-insensitive and relatively ouabain-insensitive com-
ponent of K\textsuperscript{+}-ATPase activity, and that a novel X\textsuperscript{+},K\textsuperscript{+}-ATPase α-subunit, bearing an amino terminus antigenically similar to α\textsubscript{C}, mediates the more ouabain-sensitive fraction of Na\textsuperscript{+}-independent K\textsuperscript{+}-ATPase activity. Given the fact that the ouabain sensitivities of α\textsubscript{C}β\textsubscript{b} and αβ\textsubscript{b} were virtually indistinguishable, it seems less likely that a novel β-subunit could confer ouabain sensitivity on α\textsubscript{C}. If α\textsubscript{C} indeed interacts with β\textsubscript{b} in vivo, it is logical to predict that these two subunits will be coordinately up-regulated in the colonocyte apical membrane during chronic K\textsuperscript{+} deprivation. Studies are presently under way in our laboratory to test this hypothesis.

In conclusion, the present studies show that both β\textsubscript{b} and β\textsubscript{1} can interact with α\textsubscript{C}, each creating a functional H\textsuperscript{+},K\textsuperscript{+}-ATPase that is Sch-28080-insensitive and only partially sensitive to ouabain. The functional and pharmacological properties of α\textsubscript{C}β\textsubscript{b} and αβ\textsubscript{b} holoenzymes are quite similar. Our data support the view that, in the rat distal colon, β\textsubscript{1} may play a surrogate role as the β-subunit for the colonic H\textsuperscript{+},K\textsuperscript{+}-ATPase.

REFERENCES
1. Modyanov, N. N., Mathews, P. M., Grishin, A. V., Beguin, P., Beggar, A. T., Rossier, B. C., Horisberger, J. D., and Geering, K. (1995) Am. J. Physiol. 269, C992–C997
2. Rabon, E., Gunther, R. D., Soumarmen, A., Bassilian, S., Lewin, M., and Sachs, G. (1995) J. Biol. Chem. 270, 10200–10207
3. Svedlov, E. D. (1991) Genetics 128, 91–101
4. Wing, C. S., and Smolka, A. (1995) Am. J. Physiol. 269, F1–F6
5. Shull, G. E., and Lingler J. B. (1986) J. Biol. Chem. 261, 16788–16791
6. Kone, B. C. (1996) Miner. Electrolyte Metab. 22, 349–365
7. Crowson, M. S., and Shull, G. E. (1992) J. Biol. Chem. 267, 13740–13748
8. Watanabe, T., Sato, M., Kaneko, K., Suzuki, T., Yoshida, T., and Suzuki, Y. (1991) Am. J. Physiol. 260, F803–F807
9. Jaisser, F., Rabon, E., Horisberger, J. D., Geering, K., and Rossier, B. C. (1993) J. Cell Biol. 123, 1421–1429
10. Grishin, A. V., Svedlov, V. E., Kostina, M. B., and Modyanov, N. N. (1994) FEBS Lett. 349, 144–150
11. Lee, J., Rajendran, V. M., Mann, A. S., Kashgarian, M., and Binder, H. J. (1995) J. Clin. Invest. 96, 2002–2008
12. Cougnon, M., Planzeller, G., Crowson, M. S., Shull, G. E., Rossier, B. C., and Jaisser, F. (1996) J. Biol. Chem. 271, 7277–7280
13. Foster, R. S., Jones, W. J., Hayslett, J. P., and Binder, H. J. (1985) Gastroenterology 88, 41–46
14. Cheval, L., Barlet-Bas, C., Khadouri, E., Feraile, E., Maray, S., and Doucet, A. (1991) Am. J. Physiol. 260, F800–F805
15. Ahn, K. Y., Turner, P., Madsen, K. M., and Kone, B. C. (1996) Am. J. Physiol. 270, F502–F507
16. DuBois, T. D., Codina, J., Burgos, A., and Pressley, T. A. (1995) Am. J. Physiol. 283, F500–F507
17. Ahn, K. Y., Park, K., Kim, K., and Kone, B. C. (1996) Am. J. Physiol. 271, F314–F321
18. Watanabe, T., Suzuki, T., and Suzuki, Y. (1990) Am. J. Physiol. 258, G506–G511
19. Del Castillo, J. R., Rajendran, V. M., and Binder, H. J. (1991) Am. J. Physiol. 261, G1005–G1011
20. Abrahamse, S. I., Jonge, H. R., Bindels, R. J. M., and Van Os, C. H. (1995) Biochem. Biophys. Res. Commun. 207, 1003–1008
21. Eakle, K., Lyu, R.-M., and Farley, R. A. (1995) J. Biol. Chem. 270, 1387–1389
22. Jaisser, F., Horisberger, J. D., and Rossier, B. C. (1993) Eur. J. Physiol. 425, 464–462
23. Shull, G. E. (1990) J. Biol. Chem. 265, 12123–12126
24. McDonough, A. A., Magyar, C. E., and Komatsuzaki, Y. (1994) Am. J. Physiol. 267, C991–C998
25. Marxer, A., Stiegler, B., Quarini, A., Kashgarian, M., and Hauri, H. P. (1989) J. Cell Biol. 109, 1057–1069
26. Vachon, V., Paradis, G., Marches, M., Schwartz, J. L., and Laprade, R. (1995) Biochemistry 34, 15157–15164
27. Lema, M. V., Yu, H.-Y., Takeyasu, K., Kone, B. C., and Fambrough, D. M. (1994) J. Biol. Chem. 269, 18651–18655
28. Young, R. M., Shull, G. E., and Lingrel, J. B. (1987) J. Biol. Chem. 262, 4905–4910
29. Sanford, J., Codina, J., and Birnbaumer, L. (1990) J. Biol. Chem. 266, 15707–15709
30. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
31. Parent, L., and Gopalakrishnan, M. (1995) Biophys. J. 69, 1801–1813
32. Evans, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Mol. Cell. Biol. 5, 3610–3616
33. Wallmark, B., Briving, C., Fryklund, J., Munson, K., Jackson, R., Mendlein, J., Rabon, E., and Sachs, G. (1987) J. Biol. Chem. 262, 2077–2084
34. Horisberger, J.-D., Jaisser, F., Reuben, M. A., Lasater, L. S., Chow, D. C., Forte, J. G., Sachs, G., Rossier, B. C., and Geering, K. (1991) J. Biol. Chem. 266, 19131–19134
35. Del Castillo, J. R., Sulbaran-Carrasco, M. C., and Burguillo, L. (1994) Am. J. Physiol. 266, G1083–G1089
36. Pandiyan, V., Rajendran, V. M., and Binder, H. J. (1992) Gastroenterology 102, 1846–1853
37. Jaisser, F., Escoubet, B., Coutry, N., Eugene, R., Bonvalet, P. J., and Farman, N. (1995) Am. J. Physiol. 33, C679–C687
38. Galghagan, J. M., Tan, S. S., Rahn, M. A., Curran, K. A., Campbell, W. G., Smolka, A. J., Tob, B. H., Glens, P. A., Wingo, C. S., Cain, B. D., and Van Driel, I. R. (1995) Am. J. Physiol. 268, F363–F374
39. Jaisser, F., Jazin, P., Geuring, K., Rossier, B. C., and Horisberger J. D. (1994) J. Gen. Physiol. 103, 605–623
40. Canessa, C. M., Horisberger, J. D., Louvard, D., and Rossier, B. C. (1992) EMBO J. 11, 1681–1687
41. Palasis, M., Kuntzweiler, T. A., Arguello, J. M., and Lingrel, J. B. (1996) J. Biol. Chem. 271, 14176–14182
42. Suzuki, Y., Watanabe, T., and Kaneko, K. (1993) Jpn. J. Physiol. 43, 291–298
43. Younes-Ibrahim, M., Barlet-Bas, C., Buffin-Meyer, B., Cheval, L., Rajerison, R., and Doucet, A. (1995) Am. J. Physiol. 268, F1141–F1147

Coexpression of α\textsubscript{C} with β\textsubscript{0} or β\textsubscript{1}