

Subunit Isoform Selectivity in Assembly of Na,K-ATPase α - β Heterodimers*

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Background: The Na,K-ATPase consists of one α (four isoforms) and one β (three isoforms) subunits.

Results: The α_1 preferentially assembles with β_1 , whereas α_2 preferentially binds to β_2 isoform.

Conclusion: Assembly of α - β complexes is isoform-selective.

Significance: This selectivity is crucial for cell- and tissue-specific functions of the Na,K-ATPase.

To catalyze ion transport, the Na,K-ATPase must contain one α and one β subunit. When expressed by transfection in various expression systems, each of the four α subunit isoforms can assemble with each of the three β subunit isoforms and form an active enzyme, suggesting the absence of selective α - β isoform assembly. However, it is unknown whether *in vivo* conditions the α - β assembly is random or isoform-specific. The α_2 - β_2 complex was selectively immunoprecipitated by both anti- α_2 and anti- β_2 antibodies from extracts of mouse brain, which contains cells co-expressing multiple Na,K-ATPase isoforms. Neither α_1 - β_2 nor α_2 - β_1 complexes were detected in the immunoprecipitates. Furthermore, in MDCK cells co-expressing α_1 , β_1 , and β_2 isoforms, a greater fraction of the β_2 subunits was unassembled with α_1 as compared with that of the β_1 subunits, indicating preferential association of the α_1 isoform with the β_1 isoform. In addition, the α_1 - β_2 complex was less resistant to various detergents than the α_1 - β_1 complex isolated from MDCK cells or the α_2 - β_2 complex isolated from mouse brain. Therefore, the diversity of the α - β Na,K-ATPase heterodimers *in vivo* is determined not only by cell-specific co-expression of particular isoforms, but also by selective association of the α and β subunit isoforms.

The Na,K-ATPase is universally expressed in all animal cells where it generates electrochemical ion gradients that are critical for many cellular processes (1–3). The Na,K-ATPase is composed of two essential subunits, α and β . There are four isoforms of the α subunits and three isoforms of the β subunits. In some tissues, the α - β heterodimer is associated with one of the seven members of the FXFD protein family (4, 5) that modulate kinetic properties of the enzyme.

The α_1 and β_1 isoforms are expressed ubiquitously, suggesting a housekeeping role for the α_1 - β_1 Na,K-ATPase in most cells. In contrast, other Na,K-ATPase subunit isoforms are expressed in a tissue-specific manner. The α_2 isoform is

expressed mainly in muscle and nervous system (1–3, 6, 7); the α_3 isoform is expressed mainly in neurons (8, 9), whereas the α_4 isoform is found only in testis (10, 11). The β_2 isoform is expressed predominantly in brain and muscle (3, 7), whereas the β_3 isoform is mainly expressed in lung, testis, skeletal muscle, and liver (12, 13). The α_2 isoform regulates contractility of cardiac, smooth, and skeletal muscle and plays a key role in the modulation of blood pressure in response to stress (3, 14, 15). *In vivo* human mutations in the α_2 and α_3 isoforms are associated with neurological diseases, familial hemiplegic migraine type 2, and rapid-onset dystonia-parkinsonism (16). The α_4 isoform is required for sperm motility and fertility (10, 11). The β_1 subunit plays an important role in intercellular adhesion in epithelia (17, 18), and the β_2 subunit, or adhesion molecule on glia (AMOG),² is important for adhesion and migration of neurons on glia (19). Decreased expression of the β_1 subunit is associated with cancer (reviewed in Ref. 20), whereas abnormalities in expression and distribution of the β_2 subunit are linked to glioma and epilepsy (21–24).

Therefore, it is clear that both α and β isoforms of the Na,K-ATPase have organ- and tissue-specific functions. However, very little is known about particular α - β heterodimers responsible for these roles. Transfection studies indicate that each of the four α subunit isoforms can assemble with each of the three β subunit isoforms and form a functional pump (6, 7, 25). These data imply that in cells co-expressing multiple Na,K-ATPase subunits isoforms, various α and β isoforms also assemble in different combinations, dependent on their relative cellular content. However, selective co-immunoprecipitation of the α_2 subunit but not of the ubiquitously expressed α_1 subunit, with the β_2 subunit from mouse and rat brain (19, 26), as well as from heart and adrenal medullary cells of guinea pigs and rats (26, 27) suggest that the α_2 subunit is the preferred binding partner of the β_2 subunit. In support of this hypothesis, the tissue expres-

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² The abbreviations used are: AMOG, adhesion molecule on glia; YFP- β_1 and YFP- β_2 , the fusion proteins between the yellow fluorescent protein and the Na,K-ATPase β_1 subunit and β_2 subunit, respectively; PNGase F, peptide:N-glycosidase F; Endo H, Endo- β -N-acetylglucosaminidase H; ER, endoplasmic reticulum; DOC, sodium deoxycholate; DDM, *n*-dodecyl β -D-maltoside; NTCP, sodium-taurocholate cotransporting polypeptide; MDCK, Madin-Darby canine kidney; UGGT1, UDP-glucose glycoprotein glucosyltransferase 1.

Isoform Selectivity of Na,K-ATPase α - β Heterodimers

sion pattern of the β_2 subunit, mainly in muscle and nervous system, is similar to that of the α_2 subunit (3, 7).

Here, we show that not only does the β_2 subunit preferentially assemble with the α_2 subunit but also the α_2 subunit is mostly associated with the β_2 subunit in mouse brain. In addition, by analyzing the competition of the β_1 or β_2 subunits for binding to the α_1 subunit in MDCK cells, we demonstrate that the β_1 subunit is a greatly preferred binding partner of the α_1 subunit compared with the β_2 subunit. The results of co-immunoprecipitation of α and β subunits from various detergent extracts of native tissues and cultured cells indicate that α_1 - β_1 and α_2 - β_2 heterodimers are more stable than α_1 - β_2 heterodimers. Therefore, there is selective assembly of the different α and β subunit isoforms with likely tissue-specific functional consequences.

EXPERIMENTAL PROCEDURES

Cell Lines—The Na,K-ATPase dog β_1 or human β_2 subunits linked with their N termini to YFP were constructed as described previously (28, 29). Stable MDCK cell lines expressing YFP- β_1 and YFP- β_2 were obtained and maintained as described previously (30).

Confocal Microscopy—Confocal microscopy images were acquired using the Zeiss LSM 510 laser scanning confocal microscope and LSM 510 software (version 3.2).

Primary Antibodies Used for Immunofluorescent Staining and Western Blot Analysis—For immunofluorescent staining, the monoclonal antibodies against the Na,K-ATPase α_1 subunit, clone C464.6 (Millipore) and against the Na,K-ATPase β_1 subunit, clone M17-P5-F11 (Affinity Bioreagents) and polyclonal antibodies against the Na,K-ATPase α_2 subunit, (Millipore) and against the Na,K-ATPase β_2 subunit (Millipore) were used. The polyclonal antibody against the Na,K-ATPase β_1 subunit (31), which was a generous gift of Dr. W. James Ball, Jr. (University of Cincinnati), was used for Western blot analysis. Also, the following monoclonal antibodies were used for Western blot analysis: against GFP, clones 7.1 and 13.1, which also recognizes YFP (Roche Diagnostics), against the Na,K-ATPase α_1 subunit, clone C464.6 (Millipore), against the Na,K-ATPase α_3 subunit (Upstate), against the Na,K-ATPase β_2 subunit, clone 35 (BD Bioscience Pharmingen), and against the Na,K-ATPase β_3 subunit (Santa Cruz Biotechnology).

Extraction of Proteins from MDCK Cells and Mouse Brain Homogenates—Confluent MDCK cell monolayers grown in six-well plates were rinsed twice with ice-cold PBS and incubated with 200 μ l/well of the extraction buffer at 4 °C for 30 min followed by scraping cells. Mouse brain homogenates containing 600 μ g protein in 500 μ l of 150 mM NaCl in 50 mM Tris, pH 7.5, were incubated with 500 μ l of the extraction buffer at 4 °C for 30 min. The extraction buffer contained 150 mM NaCl in 50 mM Tris, pH 7.5, and the 2 \times concentration of the indicated detergent(s). When DOC was used alone, no NaCl was added to the extraction buffer. Prior to using, the extraction buffer was mixed with Complete protease inhibitor mixture (Roche Diagnostics), 1 tablet/50 ml. Cell extracts were clarified by centrifugation (15,000 \times g, 10 min) at 4 °C. Where indicated, protein extracts were treated by PNGase F from *Flavobacterium meningosepticum* (New England Biolabs) or by Endo H from

Streptomyces plicatus (Glyco-Prozyme Inc.) according to the manufacturer's instructions prior to loading on SDS-PAGE.

Immunoprecipitation—Protein extracts from MDCK cells or from mouse brain homogenates (100–300 μ g protein) were incubated with 30 μ l of the protein A-agarose suspension (Roche Diagnostics) in a total volume 1 ml of the extraction buffer at 4 °C with continuous rotation for at least 3 h (or overnight) to remove the components that non-specifically bind to protein A. The precleared cell extract was mixed with 2 μ l of polyclonal antibodies against GFP, which also recognize YFP (Clontech), or 10 μ l of polyclonal antibodies against the Na,K-ATPase α_2 subunit (CHEMICON Intl.), or 10 μ l of polyclonal antibodies against the Na,K-ATPase α subunit (32) and incubated with continuous rotation at 4 °C for 60 min. After addition of 30 μ l of the protein A-agarose suspension, the mixture was incubated at 4 °C with continuous rotation overnight. The bead-adherent complexes were washed three times on the beads and then eluted as described previously (33).

Multi-round immunoprecipitation from MDCK cells expressing either YFP- β_1 or YFP- β_2 was performed as described above with the exception that, after the first round of immunoprecipitation using 30 μ g protein in 1% CHAPS and 5 μ l anti- α antibody, the unbound proteins in the supernatant were collected and incubated again with 5 μ l of anti- α antibody in a second round of immunoprecipitation. A third round of immunoprecipitation was performed by using 4 μ l of anti-GFP/YFP antibody from the supernatant after the second round of immunoprecipitation.

Where indicated, the bead-adherent proteins were treated with PNGase F or with Endo H. Deglycosylation by PNGase F was performed by incubation of the bead-adherent proteins with 1 μ l of PNGase F in 30 μ l of 50 mM sodium phosphate, pH 7.5, containing 1% Nonidet P-40 and at 37 °C for 1 h. Digestion by Endo H was performed by incubation of the bead-adherent proteins with 3 μ l of Endo H in 30 μ l of 50 mM sodium citrate/phosphate, pH 5.5, containing 1% Nonidet P-40 at 37 °C for 3 h. After incubation with glycosidases, the reaction mixture was separated from the beads. The adherent proteins were eluted from the beads by incubation in 30 μ l of 2 \times SDS-PAGE sample buffer for 5 min at 80 °C. To account for possible dissociation of immunoprecipitated proteins from the beads during deglycosylation, the eluted proteins were combined with the reaction mixture. After separation by SDS-PAGE, the immunoprecipitated and co-immunoprecipitated proteins were analyzed by Western blot by using appropriate antibodies.

Isolation of Basolateral Plasma Membrane Proteins of MDCK Cells Using Surface-specific Biotinylation—Cells were maintained for 6 days after becoming confluent in Transwell inserts. Biotinylation and isolations of basolateral surface proteins was performed according to procedures described previously (34–36).

Western Blot Analysis—1–10 μ g of proteins extracted from MDCK cells, microsomal membranes isolated from animal tissues in SDS-PAGE sample buffer, or 5–20 μ l of proteins eluted from the protein A-conjugated agarose beads were loaded onto 4–12% gradient SDS-PAGE gels (Invitrogen). Proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane (Bio-Rad), and detected by Western blot analysis as

described previously (33). Immunoblots were quantified by densitometry using Zeiss LSM 510 software (version 3.2).

Immunofluorescent Staining—MDCK cells were fixed by incubation with 3.75% formaldehyde in PBS for 15 min at 37 °C and permeabilized by incubation with 0.1% Triton X-100 for 5 min. Fixed cells or frozen tissue sections on FDA standard frozen tissue rat or human arrays (BioChain) were incubated with Dako protein block serum-free solution (Dako Corp.) for 30 min. Immunostaining was performed by 1-h incubation with primary antibodies followed by 1-h incubation with Alexa Fluor 633- or Alexa Fluor 488-conjugated anti-mouse or anti-rabbit antibodies (Invitrogen).

Statistical Analysis—Statistical analysis was performed using Student's *t* test (GraphPad Prism 4 software and Microsoft Excel). Statistical significance and number of experiments are specified in the figure legends.

RESULTS

Distribution of Na,K-ATPase α_1 , α_2 , β_1 , and β_2 Subunits in Rat and Human Tissues—Normal rat or human frozen tissue arrays (BioChain) that contained sections of adrenal gland, brain, breast, colon, esophagus, heart, kidney, liver, skeletal muscle, nerve, and ovary were used to perform immunofluorescent double staining of the Na,K-ATPase α_1 and α_2 subunits. The Na,K-ATPase α_1 and α_2 subunits were differentially distributed in several organs, particularly in peripheral nerves and kidney, which are shown as examples (Figs. 1 and 2A). The α_2 subunit was found predominantly in perineurium that surrounds fascicles, whereas the α_1 subunit was also present in epineurium that surrounds nerves (Fig. 1). In the kidney, the α_1 subunit was detected in tubules, whereas the α_2 subunit was seen predominantly in renal arteriole and glomerulus (Fig. 2A). Double staining of the α_1 and β_2 subunits showed that tissue distribution of the β_2 subunit is similar to that of the α_2 , but not of the α_1 subunit in both peripheral nerve and kidney sections (Figs. 1 and 2A). Also, double staining of the β_1 and β_2 subunits showed that distribution of the β_1 subunit in the nerve is similar to that of the α_1 but not of the β_2 subunit (Fig. 1B). Staining of the α_1 , α_2 , and β_2 subunits in rat brain cortex showed both co-expression of these isoforms in the same cells and cell-specific expression (Fig. 2B). Co-expression of α_1 and α_2 or α_1 and β_2 is evident from co-localization of red and green fluorescence, producing a yellow color on the merged images, whereas the differential expression of these isoforms is seen from separated green and red spots on the merged images. Co-expression of the β_1 isoform with both α_2 and β_2 isoforms in the same cells was also seen in human brain sections (data not shown).

Subunit Composition of Na,K-ATPase α - β Heterodimers in Mouse Brain—Western blot analysis detected the presence of six different isoforms of the Na,K-ATPase subunits in mouse brain extracts (Fig. 3), consistent with previously published results for rat brain (37). To validate the antibodies used to detect β subunits isoforms, we performed Western blot analysis of mouse brain extracts incubated with or without PNGase F that cleaves *N*-glycans attached to the β subunits. The bands at 33–35 kDa, which were expected for deglycosylated β_1 , β_2 , and β_3 subunits, were detected in samples treated with PNGase F by using the antibodies listed under “Experimental Procedures”

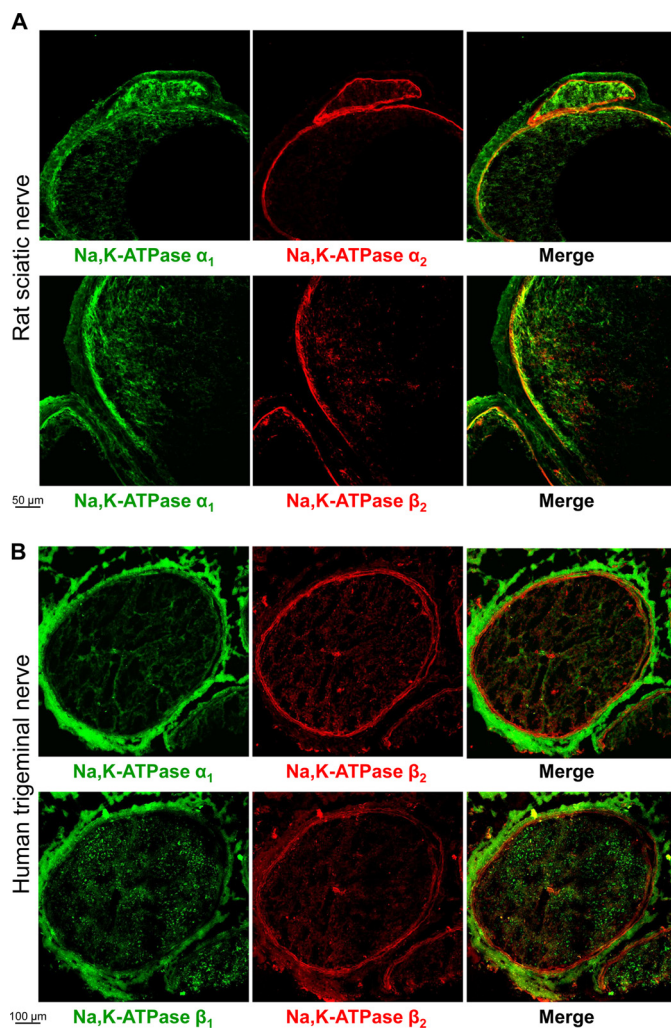


FIGURE 1. Localization of the Na,K-ATPase β_2 subunit in nerve sections is similar to that of the Na,K-ATPase α_2 subunit, but not of the Na,K-ATPase α_1 subunit or β_1 subunit. A, frozen sections of rat sciatic nerve were double stained by using mouse antibodies against α_2 subunit (green) and either rabbit antibodies against α_2 subunit (red) or rabbit antibodies against β_2 subunit (red). B, frozen sections of human trigeminal nerve were double-stained by using either mouse antibodies against α_1 subunit (green) or mouse antibodies against β_1 subunit (green) and rabbit antibodies against β_2 subunit (red). Anti-mouse Alexa Fluor 488-conjugated secondary antibodies were used to detect anti- α_1 and anti- β_1 primary antibodies, and anti-rabbit Alexa Fluor 633-conjugated secondary antibodies were used to detect anti- β_2 and anti- α_2 primary antibodies.

(Fig. 3A). In control samples, these antibodies detected bands at 45–50 kDa (Fig. 3A).

Immunoprecipitation of the β_2 subunit from 1% Nonidet P-40/0.5% DOC extracts of mouse brain homogenates resulted in co-precipitation of the α_2 isoform and of a minor amount of the α_3 isoform but not of the α_1 subunit (Fig. 3B). These results are consistent with detection of the α_2 subunit but not of the α_1 subunit in the fraction isolated from mouse brain by immunofluorescence chromatography using the antibody against the β_2 subunit (38).

Immunoprecipitation of the Na,K-ATPase α_2 subunit resulted in co-precipitation of the β_2 subunit but not of the β_1 subunit (Fig. 3C). A minor amount of the β_3 subunit was detected in the immunoprecipitated fraction (Fig. 3C). No α_1 or α_3 subunits were detected in the immunoprecipitated fraction,

Isoform Selectivity of Na,K-ATPase α - β Heterodimers

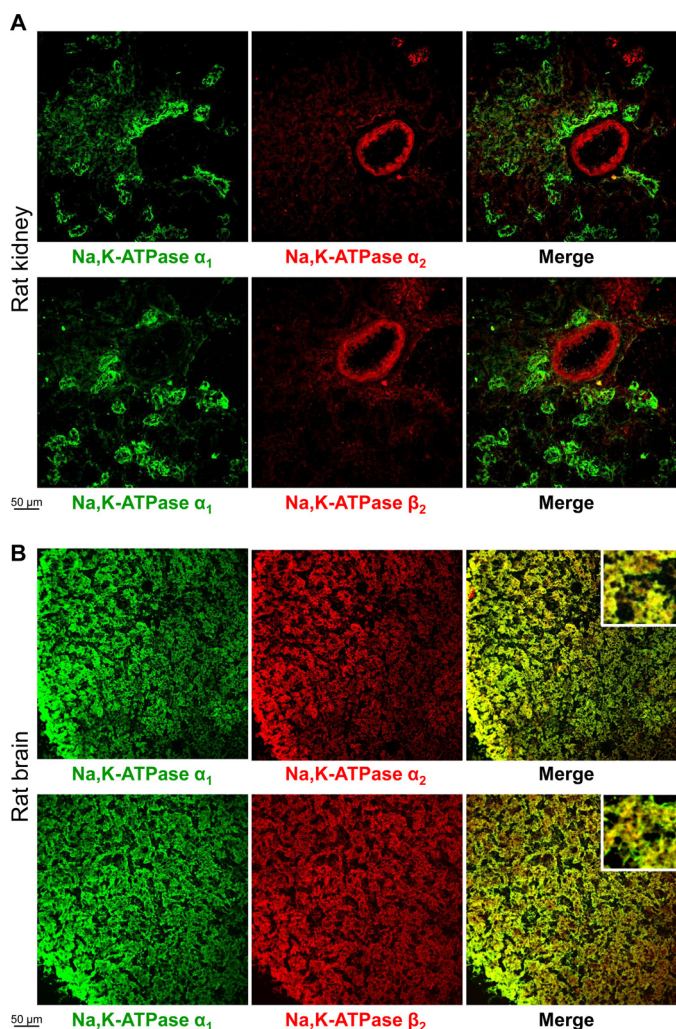


FIGURE 2. Localization of the Na,K-ATPase α_1 , α_2 , and β_2 subunits in rat kidney and brain sections. Frozen sections of rat kidney (A) and rat brain (B) were double-stained by using mouse antibodies against α_1 subunit (green) and either rabbit antibodies against α_2 subunit (red) or rabbit antibodies against β_2 subunit (red). Anti-mouse Alexa Fluor 488-conjugated secondary antibodies were used to detect anti- α_1 primary antibodies and anti-rabbit Alexa Fluor 633 conjugated secondary antibodies were used to detect anti- β_2 and anti- α_2 primary antibodies. Insets in the right panels (B) show images zoomed 5-fold.

confirming specificity of the anti- α_2 antibody. In contrast, the antibody raised against the 4/5 loop of the Na,K-ATPase α_1 subunit that is relatively homologous in other α isoforms (32) immunoprecipitated all three α isoforms, α_1 , α_2 , and α_3 and all three β subunit isoforms from mouse brain extracts (Fig. 3C), confirming its non-selectivity, as expected.

To evaluate stability of the various Na,K-ATPase α - β complexes, we compared co-immunoprecipitates of the β subunits with α subunits from mouse brain extracts obtained by using increasing concentrations of the non-ionic detergent, DDM. The amount of the α_2 subunit immunoprecipitated with specific anti- α_2 antibodies was similar in 0.5%, 1 and 2% DDM (Fig. 4A, left panel). In contrast, the amount of the β_2 subunits, which were co-precipitated with the α_2 subunit, gradually decreased with increasing detergent concentration (Fig. 4, A, left panel, and B), showing a partial dissociation of the α_2 - β_2 complex by DDM. No co-precipitation of the β_1 or β_3 subunit was found at any DDM concentration tested.

The total amount of all three α subunit isoforms immunoprecipitated by nonspecific anti- α antibodies was similar in 0.5%, 1, and 2% DDM extracts (Fig. 4A, right panel). The amount of the β_2 subunit co-precipitated by nonspecific anti- α antibodies gradually decreased with increasing detergent concentration (Fig. 4, A, right panel, and B). This decrease was similar to that observed with the specific anti- α_2 antibodies (Fig. 4, A, left panel, and B), strongly suggesting that precipitation of the β_2 subunit by nonspecific anti- α antibodies reflects α_2 - β_2 complexes. This conclusion is consistent with co-precipitation of the α_2 but not of the α_1 subunit, with the β_2 subunit (Fig. 3C). The amount of the β_1 subunit that was co-precipitated with α subunits by the nonspecific anti- α antibodies was not affected by detergent concentration (Fig. 4A, right panel). Only a slight decrease in the amount of the β_3 subunit co-precipitated with α subunits was observed with an increase in DDM concentration (Fig. 4, A, right panel, and B). These results indicate that complexes of the α_1 or α_3 subunit with either the β_1 or β_3 subunit are preserved at all tested DDM concentrations. Therefore, both α_2 and β_2 subunits predominantly associate with each other but not with other partner subunits in mouse brain.

Plasma Membrane and Intracellular Distribution of Na,K-ATPase α , β_1 , and β_2 in MDCK Cells—To test whether the α_1 subunit preferentially assembles with the β_1 or β_2 isoform, the content of α_1 -assembled and α_1 -unbound β subunits was compared in stable MDCK cell lines expressing either YFP- β_1 or YFP- β_2 . The major endogenous Na,K-ATPase isoforms in these cells are α_1 and β_1 . Both YFP- β_1 and YFP- β_2 were co-localized with the endogenous α_1 subunit in the lateral membranes, as detected by immunofluorescence (Fig. 5A), consistent with previously reported results (29). In addition, both YFP- β_1 and YFP- β_2 but not the α_1 subunit were found inside the cells (Fig. 5A), indicating that these intracellular forms of YFP- β_1 and YFP- β_2 are not assembled with the α_1 subunit. This intracellular retention was greater in dispersed cells than in confluent monolayers (Fig. 5B), and it showed co-localization with the ER marker (Fig. 5C). The fraction of YFP- β_2 localized in the ER was greater than that of YFP- β_1 (Fig. 5, A and B).

To quantify the levels of the endogenous and exogenous Na,K-ATPase subunits in the ER and the plasma membrane in the two cell lines, we analyzed total cell lysates and plasma membrane fractions by Western blot analysis. In both cell lines, the YFP-linked β subunit was detected as two bands in total lysates (Fig. 6A). These two bands represent differentially N-glycosylated species of fusion proteins. Only a single band, which corresponded to the upper band in cell lysate, was found in the basolateral membrane (Fig. 6A), indicating that the lower band in cell lysate corresponds to the intracellular fraction of YFP- β_1 or YFP- β_2 . As shown previously, this intracellular form of either YFP- β_1 or YFP- β_2 mostly represents the ER-resident fraction of each fusion protein (34). The relative amount of this ER-resident fraction appears to be greater for YFP- β_2 than for YFP- β_1 (Fig. 6A, left lanes). However, direct densitometric quantification of the two fractions of YFP- β_2 was not possible because of a significant overlap between two bands on SDS-PAGE (Fig. 6A, top panel, left lane).

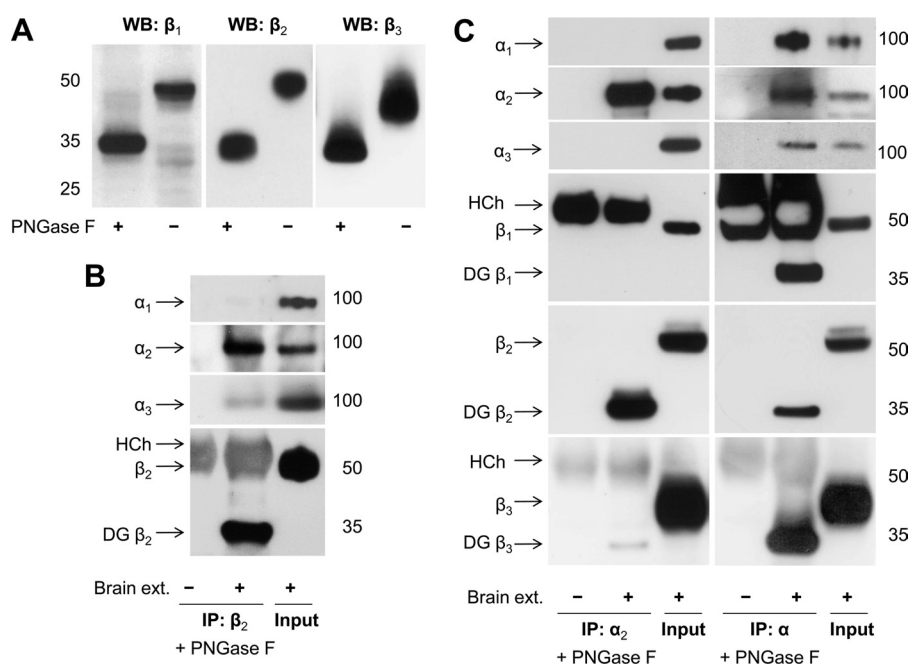


FIGURE 3. The Na,K-ATPase α_2 and β_2 subunits are selectively co-immunoprecipitated from mouse brain extracts. Proteins were extracted from mouse brain homogenate by using 1% Nonidet P-40/0.5% DOC. *A*, the antibodies against the Na,K-ATPase β_1 , β_2 , and β_3 isoforms were validated by Western blot analysis of mouse brain extracts preincubated with or without PNGase F that cleaves *N*-glycans from the β isoforms subunits and, hence, results in an increase in electrophoretic mobility of the subunits. *B*, Western blot analysis of the immunoprecipitated β_2 subunit and co-immunoprecipitated α subunit isoforms shows that the α_2 subunit is preferentially co-precipitated with the β_2 subunit. *Input* lanes contain 4 and 10% of the extract used for immunoprecipitation on α and β blots, respectively. *C*, Western blot analysis of proteins immunoprecipitated and co-immunoprecipitated by using either the α_2 -specific antibodies (*left panels*) or the α -nonspecific antibodies (*right panels*) shows selective co-immunoprecipitation of the β_2 subunit with the α_2 subunit. *Input* lanes contain 10% of the extract (*ext.*) used for immunoprecipitation. To prevent an overlap of the β subunit bands with the heavy chain band of the antibodies used for immunoprecipitation, the immunoprecipitated proteins were treated with PNGase F prior to SDS-PAGE. *HCh*, heavy chain; *IP*, immunoprecipitation; *WB*, Western blot; *DG*, deglycosylated.

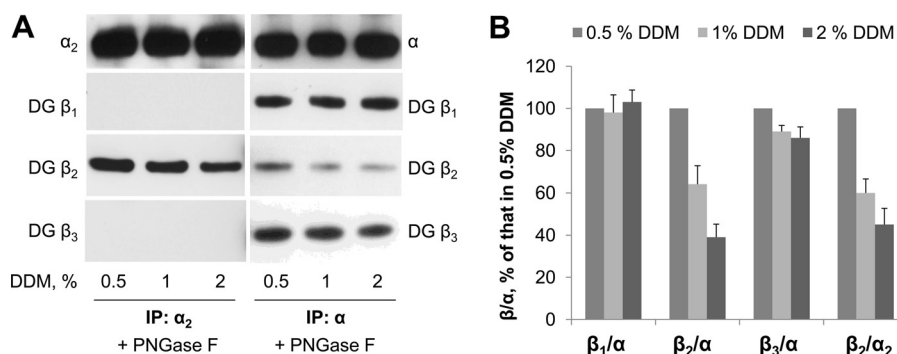


FIGURE 4. The Na,K-ATPase α_2 - β_2 complex is less stable than the Na,K-ATPase α - β_1 or α - β_3 complexes in detergent extracts obtained from mouse brain membranes. *A*, various concentrations of DDM were used to extract proteins from mouse brain homogenate. Western blot analysis of proteins immunoprecipitated (*IP*) and co-immunoprecipitated by using either the α_2 -specific antibodies (*left panels*) or the α -nonspecific antibodies (*right panels*) shows a stepwise decrease in the amount of β_2 subunits co-immunoprecipitated by using both antibodies, but not in the amount of β_1 or β_3 subunits co-immunoprecipitated by using α -nonspecific antibodies, with increasing detergent concentrations. *B*, densitometric quantification of the results shown in *A* was performed by dividing the signal from the β antibody by the corresponding signal of the α antibody. A comparative graph shows these ratios as a percentage of the ratio obtained in 0.5% DDM. *IP*, immunoprecipitation; *DG*, deglycosylated by PNGase F prior to SDS-PAGE.

To separate these two bands, we used Endo H, which is known to remove high-mannose and hybrid, but not the complex-type *N*-glycans, from glycoproteins. Treatment of total cell lysates or biotinylated proteins with Endo H resulted in a slight increase in electrophoretic mobility of the plasma membrane fraction of YFP- β_2 but not of YFP- β_1 (Fig. 6*A*). The ER-resident form in cell lysates was completely deglycosylated by Endo H, producing a band at ~60 kDa that corresponds to the protein core molecular mass of YFP- β (Fig. 6*A*). As a result, a better separation of the plasma membrane and ER fractions on SDS-PAGE was observed in Endo

H-treated cell lysates, allowing densitometric quantification (Fig. 6*B*).

This quantification confirmed the greater ER retention of YFP- β_2 (49% of total cellular content) as compared with YFP- β_1 (31% of total cellular content) in mature cell monolayers. The difference in the relative content of the ER form of YFP- β_2 and YFP- β_1 was more prominent in immature cell monolayers, 80 and 38% of total cellular content, respectively (Fig. 6, *A* and *B*). In contrast, the levels of YFP- β_1 and YFP- β_2 in the basolateral membrane were similar in the two transfected cell lines (Fig. 6*C*). Also, the levels of the endogenous α_1 and β_1 subunits were

Isoform Selectivity of Na,K-ATPase α - β Heterodimers

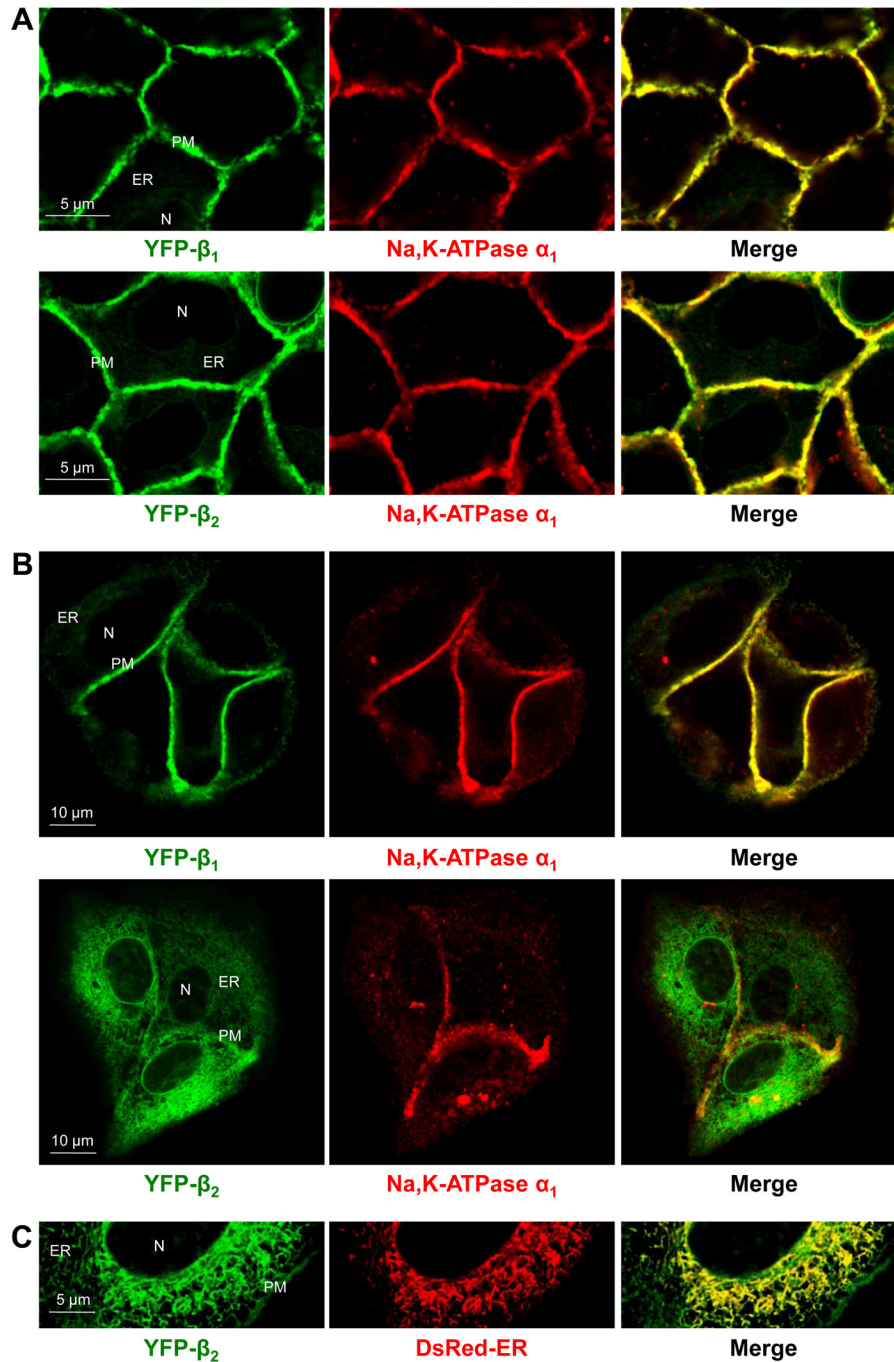


FIGURE 5. The amount of α_1 -unbound YFP- β_2 retained in the ER of MDCK cells is greater than that of YFP- β_1 . Horizontal confocal microscopy sections of confluent monolayers (A) or dispersed colonies (B and C) of MDCK cells expressing either YFP- β_1 or YFP- β_2 . Both YFP- β_1 and YFP- β_2 (green) are co-localized with the endogenous α_1 subunit (red) in the lateral membranes, but not inside the cells as detected by immunostaining of fixed cells using the monoclonal antibody against the Na,K-ATPase α_1 subunit (A and B). The intracellular retention of α_1 -unassembled YFP- β_2 is more prominent than that of YFP- β_1 , and more evident in dispersed colonies than in confluent monolayers. This intracellular fraction of YFP- β_2 (green) shows co-localization with the ER (red) as detected by transient expression of the fluorescent ER marker, DsRed2-ER (C). N, nucleus; PM, plasma membrane.

similar in YFP- β_1 - and YFP- β_2 -expressing MDCK cell lines (Fig. 6C).

Therefore, the amount of α_1 -unassembled YFP- β_2 in the ER is significantly greater than that of YFP- β_1 , whereas the quantities of α_1 -assembled YFP- β_1 and YFP- β_2 in the plasma membrane are similar (Fig. 5 and see Fig. 8), suggesting preferential assembly of the α_1 subunit with the β_1 isoform rather than with the β_2 isoform.

Stability of Na,K-ATPase α_1 - β_1 and α_1 - β_2 Complexes Isolated from MDCK Cells—Co-immunoprecipitation of the Na,K-ATPase α_1 subunit with YFP- β_1 was detected in 1% Triton X-100, 1% DDM, 1% Nonidet P-40, 1% digitonin, and also in a mixture of 1% Nonidet P-40 and 0.5% DOC (Fig. 7A). The amount of co-precipitated α_1 subunit was similar in all tested detergents. In contrast, co-immunoprecipitation of the Na,K-ATPase α_1 subunit with YFP- β_2 was observed only in a few

Isoform Selectivity of Na,K-ATPase α - β Heterodimers

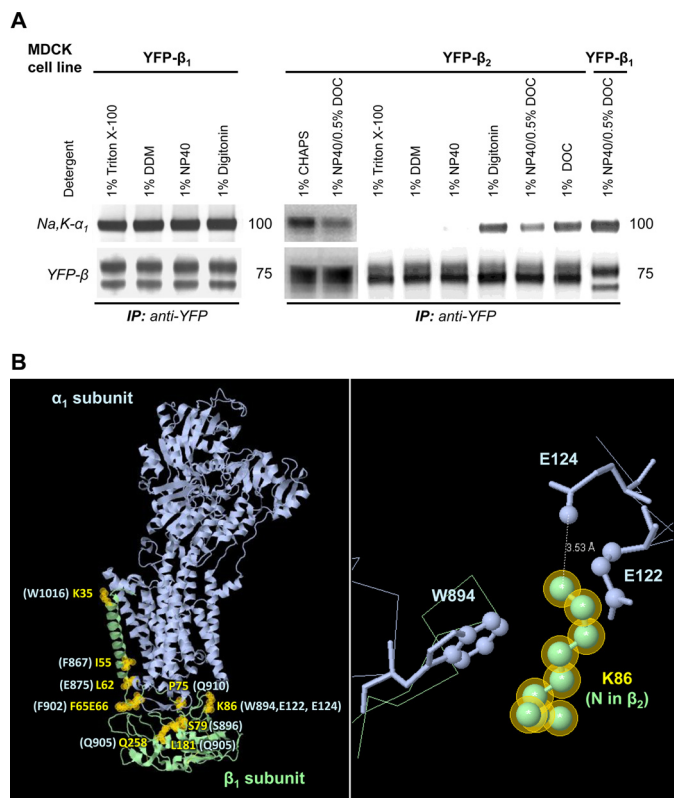


FIGURE 7. The Na,K-ATPase α_1 - β_1 complex is more stable than the Na,K-ATPase α_1 - β_2 complex in detergent extracts from MDCK cells. *A*, MDCK cells stably expressing either YFP- β_1 or YFP- β_2 were lysed by incubation with the extraction buffer containing an appropriate detergent (as indicated). After scraping the cells and removing non-extracted material by centrifugation, YFP-linked β_1 or β_2 subunits were immunoprecipitated. Immunoprecipitated YFP- β_1 or YFP- β_2 and co-immunoprecipitated α_1 subunit were analyzed by Western blot. Co-immunoprecipitation of the Na,K-ATPase α_1 subunit with YFP- β_1 was detected in all tested detergents. In contrast, co-immunoprecipitation of the Na,K-ATPase α_1 subunit with YFP- β_2 was observed only in selected detergents. *B*, a model of the Na,K-ATPase α_1 and β_1 subunits based on the crystal structure of the sodium-potassium pump at 2.4 Å resolution (Protein Data Bank code 2ZXE) (39) shows the α_1 -interacting residues in the β_1 subunit that are different in the β_2 subunit (yellow labels and yellow shaded circles). The corresponding β_1 -interacting residues in the α_1 subunit are indicated in light blue. A close-up view of Lys-86 of the β_1 subunit and its interacting residues in the α_1 subunit is shown in the right panel.

subunits. Co-precipitated fractions of YFP- β_1 or YFP- β_2 contained predominantly mature forms and minor amounts of the ER-resident immature forms (Fig. 8, *C* and *D*). The second round of immunoprecipitation using anti- α antibody precipitated the rest of the α_1 subunits and mature YFP- β_1 or YFP- β_2 . The third round of immunoprecipitation using anti-GFP antibody pulled down almost exclusively the immature forms of YFP- β_1 or YFP- β_2 and no α_1 subunits (Fig. 8, *C* and *D*). These results confirm that both YFP- β_1 and YFP- β_2 are assembled with the α_1 subunits in the plasma membrane, whereas the majority of the ER-resident YFP- β_1 and YFP- β_2 are not assembled with the α_1 subunits (Fig. 8, *C* and *D*). In addition, the data demonstrate that the (YFP- β_2)- α_1 complex is fully preserved in 1% CHAPS extracts.

DISCUSSION

β_1 , but Not β_2 Isoform, Is Preferred Binding Partner of α_1 Subunit—YFP- β_1 and YFP- β_2 stably expressed in MDCK cells are distributed predominantly between the basolateral plasma

membrane and ER (Fig. 5). All of the β subunits present in the basolateral membrane are α_1 -assembled, as we showed previously for the endogenous β_1 subunits (40) and now for both exogenous YFP- β_1 and YFP- β_2 (Fig. 8). In contrast, the majority of the ER-resident β subunits are not bound to α_1 subunits (Fig. 5*B* and Fig. 8, *C* and *D*) (34, 40). These results are consistent with the previous finding showing that both YFP- β_1 and YFP- β_2 compete with the endogenous β_1 subunits for binding to the limited amount of the endogenous α_1 subunits (34). Only the α_1 -assembled β subunits exit the ER, whereas the unassembled subunits are retained in the ER and rapidly degraded (34). As a result of this competition, a fraction of α_1 - β_1 heterodimers exported from the ER is replaced by α_1 -(YFP- β) heterodimers, explaining the decrease in the amount of the mature endogenous β_1 subunit in the basolateral membrane in both YFP- β_1 - and YFP- β_2 -expressing cell lines as compared with non-transfected MDCK cells (Fig. 6*C*). YFP- β_1 - and YFP- β_2 -expressing cell lines have similar quantities of exogenous and endogenous Na,K-ATPase subunits in the plasma membrane (Fig. 6*C*), whereas the abundance of the ER-located α_1 -unbound YFP- β_2 is greater than that of YFP- β_1 (Fig. 5 and Fig. 6, *A* and *B*). Because the ER retention of YFP- β_1 or YFP- β_2 is due to their competition with the same number of endogenous β_1 subunits for binding to the α_1 subunit, these results imply that the β_2 subunit has lower affinity for the α_1 subunit than does the β_1 subunit.

This interpretation, however, is complicated by the fact that α_1 -unassembled β subunits and β -unassembled α_1 subunits are not freely floating in the ER. Instead, the orphan α and β subunits are bound to ER chaperones (33, 41), which facilitate normal folding of the subunits and possibly the assembly process *per se*. The β_2 but not the β_1 subunit persistently binds calnexin in the ER, suggesting that it undergoes repeated calnexin-assisted folding prior to its assembly with the α subunit (29, 33). Persistent calnexin binding to glycoproteins is dependent on repeated cycles of de- and reglucosylation of glycoprotein *N*-glycans by the ER glucosidase and UGGT1, respectively (42). It is possible that the β_2 subunit is not completely folded by the time when calnexin is dissociated from deglycosylated β_2 subunit and thus is recognized by the folding sensing enzyme, UGGT1 (43). UGGT1 reglucosylates the β_2 subunit *N*-glycans, which results in repeated calnexin binding. However, it cannot be excluded that calnexin-free β_2 subunits bind UGGT1 not because they are misfolded, but because they fail to assemble with the α_1 subunits due to their lower α_1 -binding affinity as compared with that of the β_1 subunits. The UGGT1-mediated reglucosylation of the β_2 subunit would then induce its re-binding to calnexin. Therefore, both longer association with calnexin and greater accumulation in the ER of the β_2 subunit as compared with those of the β_1 subunit could result from either the longer time required for folding of the β_2 subunit or its lower affinity to the α_1 subunit. The lower binding affinity of the β_2 subunit toward the α_1 subunit is anticipated from the differences between the α_1 -interacting residues of the β_1 subunit and the corresponding residues of the β_2 subunit (Fig. 7*B*).

The α_1 - β_2 heterodimers are less resistant to the disruptive effect of various detergents than the α_1 - β_1 complexes. Co-immunoprecipitation of the Na,K-ATPase α_1 subunit with

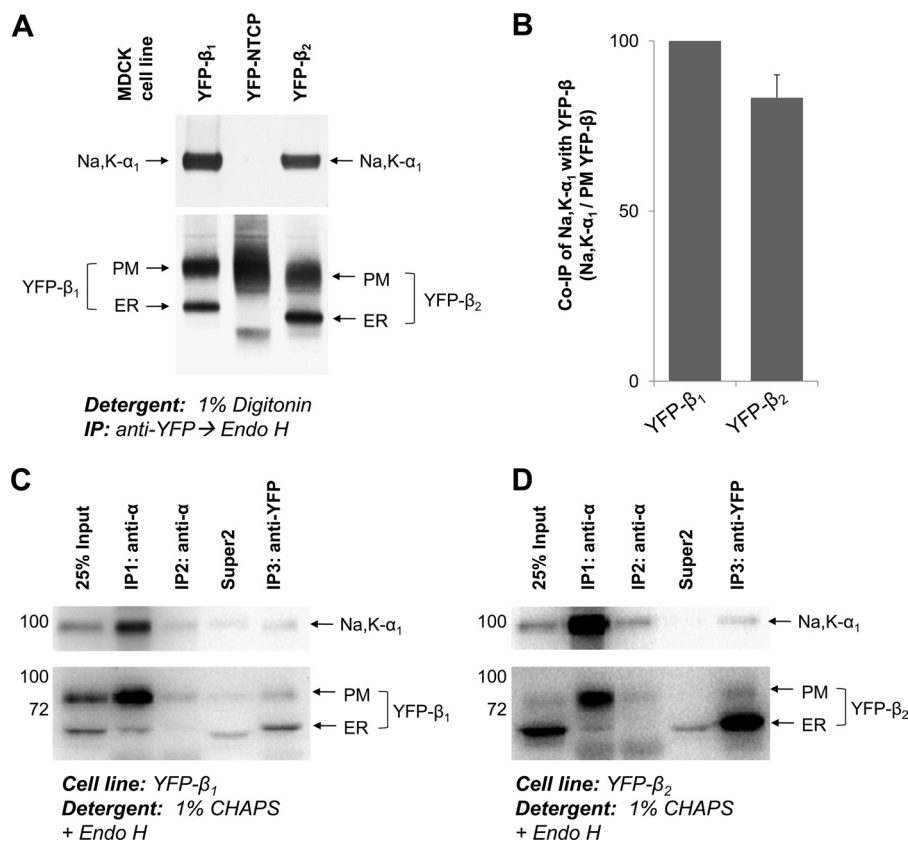


FIGURE 8. The Na,K-ATPase α_1 - β_2 complex is preserved in digitonin and CHAPS extracts from MDCK cells. *A*, proteins were extracted from MDCK cells expressing YFP- β_1 , or YFP- β_2 , or YFP-linked bile acid transporter (YFP-NTCP), with the extraction buffer containing 1% digitonin. YFP-linked β_1 or β_2 subunits were immunoprecipitated and treated with Endo H followed by elution of proteins from the beads. Co-immunoprecipitation of the Na,K-ATPase α_1 subunit was detected with YFP- β_1 and with YFP- β_2 , but not with YFP-NTCP, indicating that there is no nonspecific precipitation of the α_1 subunit. *B*, densitometric quantification of the results presented in *A* was performed by dividing the density of the α_1 subunit band by the density of the corresponding PM YFP- β band. *C* and *D*, proteins were extracted from MDCK cells expressing either YFP- β_1 (*C*) or YFP- β_2 (*D*) using extraction buffer containing 1% CHAPS and subject to successive rounds of immunoprecipitation (IP) using anti- α antibody followed by a final round of immunoprecipitation using anti-GFP/YFP antibody. Both extracted and immunoprecipitated proteins were treated with Endo H prior to SDS-PAGE. The majority of YFP- β subunits co-immunoprecipitated with α_1 were complex-type glycosylated. Almost all of the β subunits not assembled with α_1 were Endo H-sensitive and immunoprecipitated in the final anti-GFP/YFP immunoprecipitation. PM, plasma membrane; Super2, supernatant after the second round of immunoprecipitation.

YFP- β_2 was observed only in selected detergents, and the amount of co-precipitated α_1 subunit varied in these detergents (Fig. 7), showing that the α_1 - β_2 complex is partially or completely disrupted by the majority of tested detergents. On the other hand, the amount of the Na,K-ATPase α_1 subunit that co-precipitated with YFP- β_1 is the same in all tested detergents, indicating that the α_1 - β_1 complex is fully preserved in these detergents (Fig. 7). These results are in agreement with previously reported disruption of α_1 - β_2 , but not of the α_1 - β_1 or α_2 - β_2 complexes formed in *Xenopus* oocytes by Triton X-100 (44, 45).

Selective Formation of α_2 - β_2 Na,K-ATPase in Mouse Brain—The β_2 subunit of the Na,K-ATPase was first discovered as AMOG (19). Immunoaffinity purification of AMOG from mouse brain by using AMOG-specific antibody resulted in co-purification of a 100-kDa protein that later was identified as the Na,K-ATPase α_2 subunit (and possibly α_3 subunit), but not the α_1 subunit (38). Consistent with these data, we found that immunoprecipitation of the β_2 subunit from mouse brain resulted in co-immunoprecipitation of the α_2 , but not of the α_1 subunit (Fig. 3*B*). Conversely, immunoprecipitation of the α_2 subunit selectively co-precipitated the β_2 subunit (Fig. 3*C* and Fig. 4). Six isoforms of the Na,K-ATPase are expressed in the brain (9, 37). Both α_2 and β_2 subunits are expressed predomi-

nantly in glial cells (37), so the formation of the α_2 - β_2 complexes is in part due to the cell-specific co-expression of the two isoforms. However, both α_2 and β_2 subunits are also found in subsets of neurons (37). Similarly, the α_1 and β_1 isoforms are expressed in both glial cells and neurons. With the exception of the neuron-specific α_3 subunit, other isoforms are expressed in both neurons and glial cells (37, 46–48). Even though there are cell- and region-specific differences in expression of various isoforms, many cell types in brain contain multiple Na,K-ATPase subunit isoforms (9, 37, 46). Accordingly, co-expression of the α_1 , α_2 , and β_2 subunits in the same cells in rat brain cortex is detected here by immunofluorescence (Fig. 2*B*). Therefore, the preferential formation of the α_2 - β_2 complexes in the brain is determined not only by cell-specific co-expression of these isoforms, but also by their binding preferences. Preferential formation of the α_2 - β_2 was also detected in heart and adrenal medullary cells, where the α_1 subunit is more abundant than the α_2 subunit (26, 27), emphasizing preferential β_2 subunit binding to the α_2 subunit.

Interestingly, the α_2 - β_2 complexes are less stable than complexes of the α_1 or α_3 subunit with either β_1 or β_3 subunit (Fig. 4). Recent studies have demonstrated that α_2 - β_1 complexes are less stable to heat and detergents than α_1 - β_1 or α_3 - β_1 com-

Isoform Selectivity of Na,K-ATPase α - β Heterodimers

plexes perhaps due to weaker interactions of the α_2 subunit with phosphatidylserine, which stabilizes the protein (49). Thus, it is possible that detergent-mediated disruption of α_2 - β_2 complexes (Fig. 4) is the result of displacement of selectively bound phosphatidylserine.

Preferential assembly of α_2 and β_2 isoforms in the brain may have several implications. The β subunits are known to modify the kinetic properties of the α isoforms. The α_2 - β_2 heterodimer has the lowest K^+ affinity among nine different α - β heterodimers formed by each of the three α subunit isoforms (α_1 - α_3) and each of the three β subunit isoforms (β_1 - β_3) (25). Thus, assembly of the α_2 subunit preferentially with the β_2 isoform may be crucial to restore external K^+ homeostasis after a series of action potentials in the nervous system because the α_2 - β_2 heterodimer would respond to an increase in external K^+ because of its low K^+ affinity (25). In astrocytes, the Na,K-ATPase α_2 subunits form complexes with different glutamate transporters, and glutamate inward transport is inhibited by ouabain, suggesting a specific role of the Na,K-ATPase α_2 subunit in reuptake of glutamate from the synaptic cleft (50). Because the activity of the least K^+ -sensitive α_2 - β_2 isoform would increase more at the elevated external K^+ concentration, assembly with the β_2 subunit may be important for the specific role of the Na,K-ATPase α_2 subunit in glutamate clearance.

Considerable evidence exists for the presence of endogenous ouabain-like molecules in mammalian tissues that may serve to regulate Na,K-ATPase activity (3, 14, 51). Particularly, a signaling role of the Na^+/K^+ -ATPase has been demonstrated in regulating synaptic plasticity and dendritic growth in cortical neurons (52). The human α_1 , α_2 , and α_3 isoforms have similar ouabain affinities (25). However, the lowest K^+ affinity of the α_2 - β_2 isoform implies that this heterodimer has the lowest K^+ /ouabain antagonism as compared with other α - β heterodimer isoforms (25). So, at physiological K^+ concentrations, ouabain and endogenous ouabain-like compounds may bind predominantly to the α_2 - β_2 isoform and to a lesser extent to other complexes and thus specifically regulate the α_2 -dependent signaling pathways.

Natural *in vivo* mutations in the α_2 subunit are associated with familial hemiplegic migraine and epilepsy (16). Most of these mutations cause functional defects in active Na^+ and K^+ transport and impaired clearance of extracellular K^+ or glutamate due to either the impairment of maturation and hence plasma membrane delivery of the enzyme, or the loss of the catalytic activity (16, 53). It is known that neurological diseases, particularly epilepsy, are closely associated with the ER stress-related retention of essential ion transporters in the ER (54–57). We showed recently that the β_2 isoform is much more sensitive to the ER stress than the β_1 isoform (33). Because the β subunit is essential for maturation of the Na,K-ATPase α - β heterodimers (58) and the α_2 selectively forms a complex with the β_2 isoform in the brain, it is possible that stress-induced impairment of the β_2 subunit folding in the ER increases the ER retention of the α_2 subunit, which decreases the Na,K-ATPase ion transport activity of the $\alpha_2\beta_2$ Na,K-ATPase and thus contributes to epilepsy. Consistent with this hypothesis, abnormalities in distribution of the β_2 subunit are linked to epilepsy (21, 22).

Therefore, the selectivity of α - β assembly, which is determined both by cell-specific expression and by isoform-specific binding preferences of α and β subunits, is crucial for cell- and tissue-specific functions of the Na,K-ATPase.

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