Accelerated Publications

Antisera Specific for the α1, α2, α3, and β Subunits of the Na,K-ATPase: Differential Expression of α and β Subunits in Rat Tissue Membranes†

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ABSTRACT: We have developed a panel of antibodies specific for the α1, α2, α3, and β subunits of the rat Na,K-ATPase. TrpE–α subunit isoform fusion proteins were used to generate three antisera, each of which reacted specifically with a distinct α subunit isotype. Western blot analysis of rat tissue microsomes revealed that α1 subunits were expressed in all tissues while α2 subunits were expressed in brain, heart, and lung. The α3 subunit, a protein whose existence had been inferred from cDNA cloning, was expressed primarily in brain and copurified with ouabain-inhibitable Na,K-ATPase activity. An antiserum specific for the rat Na,K-ATPase β subunit was generated from a TrpE–β subunit fusion protein. Western blot analysis showed that β subunits were present in kidney, brain, and heart. However, no β subunits were detected in liver, lung, spleen, thymus, or lactating mammary gland. The distinct tissue distributions of α and β subunits suggest that different members of the Na,K-ATPase family may have specialized functions.

The Na,K-ATPase[1] is the enzymatic activity responsible for the active transport of Na^+ and K^+ in most eukaryotic cells. The enzyme has been shown to consist of two subunits. The α or catalytic subunit contains the site for ATP hydrolysis (Cantley, 1981). The β subunit is a glycosylated polypeptide whose function has not yet been established.

The α subunit of the Na,K-ATPase is encoded by a multigene family. Three α subunit genes have been localized to different chromosomes in the mouse (Kent et al., 1987), and cDNA clones for three rat α subunit isoforms (α1, α2, α3) have been characterized (Shull et al., 1986; Herrera et al., 1987). To date, a single form of the rat Na,K-ATPase β subunit has been identified (Mercer et al., 1986; Young et al., 1987). Two biochemically distinct isoforms of the α subunit (α and α+) have been described in the rat (Sweadner, 1979). The α (α1) form is the predominant species found in kidney (Sweadner, 1979), whereas the α and α+ (α2) forms are present in brain (Sweadner, 1979), heart (Sweadner & Farshi, 1987), and adipose tissue and skeletal muscle (Lytton et al., 1985). The tissue distribution of the αIII (α3) subunit has not been determined as yet.

It is generally agreed that the molar ratio of α/β subunits in the Na,K-ATPase holoenzyme is 1:1 (Cantley, 1981). However, hybridization analysis has revealed marked differences in α and β subunit mRNA levels in many rat tissues (Emanuel et al., 1987; Orlowski & Lingrel, 1988). In adult liver (Emanuel et al., 1987) and lung and muscle (Orlowski & Lingrel, 1988), the level of β subunit mRNA is substantially lower than the level of expression of any of the α subunit mRNAs. The marked differences in the level of expression of α and β subunit mRNAs suggest that translational or posttranslational control mechanisms may serve to maintain equimolar amounts of both Na,K-ATPase subunits.

The ability to characterize expression of Na,K-ATPase subunit polypeptides has been difficult, primarily because antibodies specific for each α subunit isoform have not been available. Although antisera reactive with the α and α+ subunits have been used to examine tissue and developmental expression of the Na,K-ATPase [reviewed by Sweadner (1989)], the conclusions derived from such experiments must be reevaluated in view of the fact that in some rat tissues the α+ isoform may represent a mixture of both the α2 and α3 subunits (Sweadner, 1989). Distinguishing the α2 from the α3 form of the α subunit is further complicated by the fact that the in vitro translation products of α2 and α3 mRNAs exhibit similar mobilities on SDS–PAGE (Schneider et al., 1988).

To further investigate the tissue distribution of Na,K-ATPase polypeptides, we have developed a panel of antibodies specific for the three known rat α subunit isoforms. Antisera against the one known β subunit of the rat Na,K-ATPase has also been generated. Although a putative isoform of the rat β subunit (β2) has recently been identified (Martin-Vasallo et al., 1989), it is not known whether our β subunit antisera are reactive with this polypeptide. Polyclonal rabbit antisera were raised against TrpE–rat α1, α2, α3, and β subunit fusion proteins. Antisera produced from a specific α subunit fusion protein contain antibodies which are monospecific for that isoform and can be purified by immunoabsorption. Examination of rat tissue microsomal fractions revealed substantial differences in the tissue distribution of α and β subunit polypeptides. The α1 subunit was present in all tissues tested while the α2 subunit was detected in brain, heart, and lung. Expression of the α3 subunit was restricted primarily to brain. β subunit polypeptides were present in kidney, brain, and heart but were undetectable in liver, spleen, lung, thymus, and lactating mammary gland. These results raise interesting questions regarding the functional significance of Na,K-AT-

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[1] Abbreviations: Na,K-ATPase, Na^+,K^+-activated adenosinetriphosphate phosphohydrolase (EC 3.6.1.3); bp, base pair(s); PBS, phosphate-buffered saline; SDS–PAGE, sodium docecyl sulfate–polyacrylamide gel electrophoresis. The nomenclature used to describe Na,K-ATPase α subunit isoforms is in accordance with Felsenfeld and Sweadner (1988).

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Pase isoform diversity and the overall control of Na,K-ATPase expression.

Experimental Procedures

Plasmid Constructions. cDNA fragments specific for the rat Na,K-ATPase α1, α2, α3, and β subunits were fused to the *Escherichia coli* trpE gene as follows:

(*a*) *α1 Subunit.* A cDNA clone containing the entire coding region of the rat α1 subunit (Emanuel et al., 1988) was digested with *Bal*I and *Bam*HI. The 526-bp fragment was ligated to pATH11 (provided by T. Koerner), which had been digested with *Sma*I and *Bam*HI. The resulting plasmid construction (pFPα1) fused the TrpE protein to 175 amino acids of the α1 subunit protein, which correspond to residues 338–513 of the sequence deduced from cDNA.

(*b*) *α2 Subunit.* A full-length rat α2 subunit cDNA (Emanuel et al., 1987) was digested with *Bal*I and *Rsa*I. The 553-bp fragment was ligated to pATH11, which had been digested with *Sma*I. The resulting plasmid construction (pFPα2) fused the TrpE protein to 184 amino acids of the α2 subunit protein, which correspond to residues 335–519 of the sequence deduced from cDNA.

(*c*) *α3 Subunit.* Full-length rat α3 subunit cDNA (Emanuel et al., 1987) was digested with *Bal*I and *Pst*I. The 583-bp fragment was ligated to pATH11, which had been digested with *Sma*I and *Pst*I. The resulting plasmid construction (pFPα3) fused the TrpE protein to 194 amino acids of the α3 subunit, which correspond to residues 320–514 of the sequence deduced from cDNA.

(*d*) *β Subunit.* Rat β subunit cDNA clone rb19G (Mercer et al., 1986) was digested with *Hin*dIII to liberate a DNA fragment containing 710 bp of β subunit cDNA and 29 bp of pAT153 DNA (from the *Eco*RI site to *Hin*dIII site). The 739-bp fragment was ligated to pATH3 (provided by T. Koerner), which had been digested with *Hin*dIII. The resulting plasmid construction (pFPβ1) fused the TrpE protein to 152 amino acids of the β subunit, which correspond to amino acids 152–304 of the sequence deduced from cDNA.

Preparation of Fusion Proteins and Rabbit Immunizations. Fusion proteins were produced in *E. coli* strain DH-1 grown in M9CA medium containing ampicillin (100 μg/mL) and tryptophan (40 μg/mL). Induction with 3β-indolacrylic acid, harvesting, and initial purification of TrpE fusion proteins were as described by Dieckmann and Tzagoloff (1985). Total bacterial protein was solubilized at 37 °C in cracking buffer [6 M urea, 1% β-mercaptoethanol, phosphate-buffered saline (PBS), pH 7.2] (P. Novick, personal communication) and size fractionated by preparative SDS–PAGE. Fusion proteins of the expected size were visualized by immersion of the gels in ice-cold 2.5 M KCl with the appropriate band then being excised. The acrylamide gel slices were rinsed three times for 5 min in distilled water, homogenized with a Tekmar homogenizer, and passed through a 27-gauge needle.

For initial rabbit immunizations, 0.5 mL of gel slurry was emulsified with 0.5 mL of Freund's complete adjuvant and injected into multiple back sites of New Zealand White rabbits. Each of the fusion proteins was used to immunize a set of two rabbits. Rabbits received additional intradermal boosts every 2 weeks thereafter of 0.5 mL of gel slurry emulsified with 0.5 mL of incomplete Freund's adjuvant. After the third boost, aliquots of serum were assayed for Na,K-ATPase subunit reactivity by immunoblotting.

Antibody Purification. Antibodies specific for each Na,K-ATPase α subunit isoform were purified according to the following protocol. Total cellular protein from 100-mL bacterial cultures was solubilized in cracking buffer. Proteins were size fractionated by SDS–PAGE and transferred to nitrocellulose (Towbin et al., 1979). Proteins were visualized by Ponceau-S staining, and a strip of nitrocellulose containing the fusion protein band was removed. FPα1, FPα2, or FPα3 antisera were diluted 1:10 in PBS and incubated with nitrocellulose strips containing each of the other fusion proteins to remove cross-reactive antibodies. (For example, FPα1 antiserum was absorbed against nitrocellulose strips containing FPα2 and FPα3 fusion proteins.) Strips were allowed to absorb antisera for 1 h, then removed, washed in 0.1 M glycine hydrochloride buffer (pH 2.5) for 2 min, neutralized by three washes in PBS, and then reapplied to the antisera. The above process was repeated seven times.

Rat Tissue Microsomes and Immunoblotting. Tissues were excised from 2-week-old and adult Sprague-Dawley rats. Mammary tissue was removed from lactating female rats 1 week postpartum. Crude microsomal membrane fractions were prepared by the method of Jorgenson (1974) and protein concentrations determined as described by Bradford (1976). Solubilized microsomal proteins were fractionated on SDS-containing 7.5% or 10% polyacrylamide gels (40 μg of protein/lane) and transferred to nitrocellulose sheets essentially as described (Towbin et al., 1979). The nitrocellulose was quenched for 1 h in blotto (5% dry milk, 3% goat serum, and 0.5% Tween 20 in PBS) followed by incubation with Na,K-ATPase subunit antiserum for 16 h. Blots were rinsed three times with wash buffer (PBS, 0.5% Tween 20) and then incubated with alkaline phosphatase conjugated goat anti-rabbit (second) antibody (Kirkegaard and Perry Laboratories) for 1 h. Blots were rinsed three times with wash buffer and developed as previously described (Blake et al., 1984).

Rat brain microsomes were enriched for ouabain-inhibitable Na,K-ATPase activity as described by Sweadner (1978). Briefly, a crude rat brain microsomal membrane fraction was extracted with SDS, layered over a 7–30% (w/v) linear sucrose gradient, and centrifuged at 27 000 rpm for 6 h in a Beckman SW28 rotor. Fractions of 2 mL were collected from the bottom of the tube, any pelleted material being avoided. Each fraction was assayed for protein concentration by the method of Bradford (1976) and ouabain-inhibitable Na,K-ATPase activity by the procedure described by Post and Sen (1967).

Results

Antisera Specific for the α1, α2, α3, and β Subunits of Rat Na,K-ATPase. The strategy adopted for the production of Na,K-ATPase α and β subunit antibodies is outlined in Figure 1. Segments of the cDNAs encoding each of the three isoforms of the rat α subunit were fused to the 3′ terminus of the *E. coli* trpE gene by using the expression vector pATH11. cDNA fragments spanning a common, overlapping portion of the α subunit (panel A) were used to generate fusion proteins FPα1, FPα2, and FPα3 (panel B). The trpE–α subunit constructs fuse approximately 20 kDa of α subunit protein to the 37 kDa of TrpE protein and produce proteins of the expected size, as shown in panel C. A cDNA fragment encoding amino acids 152–304 of the rat Na,K-ATPase β subunit was fused to extend the reading frame of trpE by using the expression vector pATH3. The trpE–β subunit construct FPβ1 (Figure 1, panel A), resulted in the fusion of the carboxyl-terminal 17 kDa of the β subunit to 37 kDa of TrpE protein (panel B). As shown in panel C, a novel fusion protein of the expected size was produced from the trpE–β subunit expression vector pFPβ1.

To prepare antisera specific for each α subunit isoform, antiserum raised against a TrpE–α subunit isoform fusion protein was immunoabsorbed to the other α subunit fusion

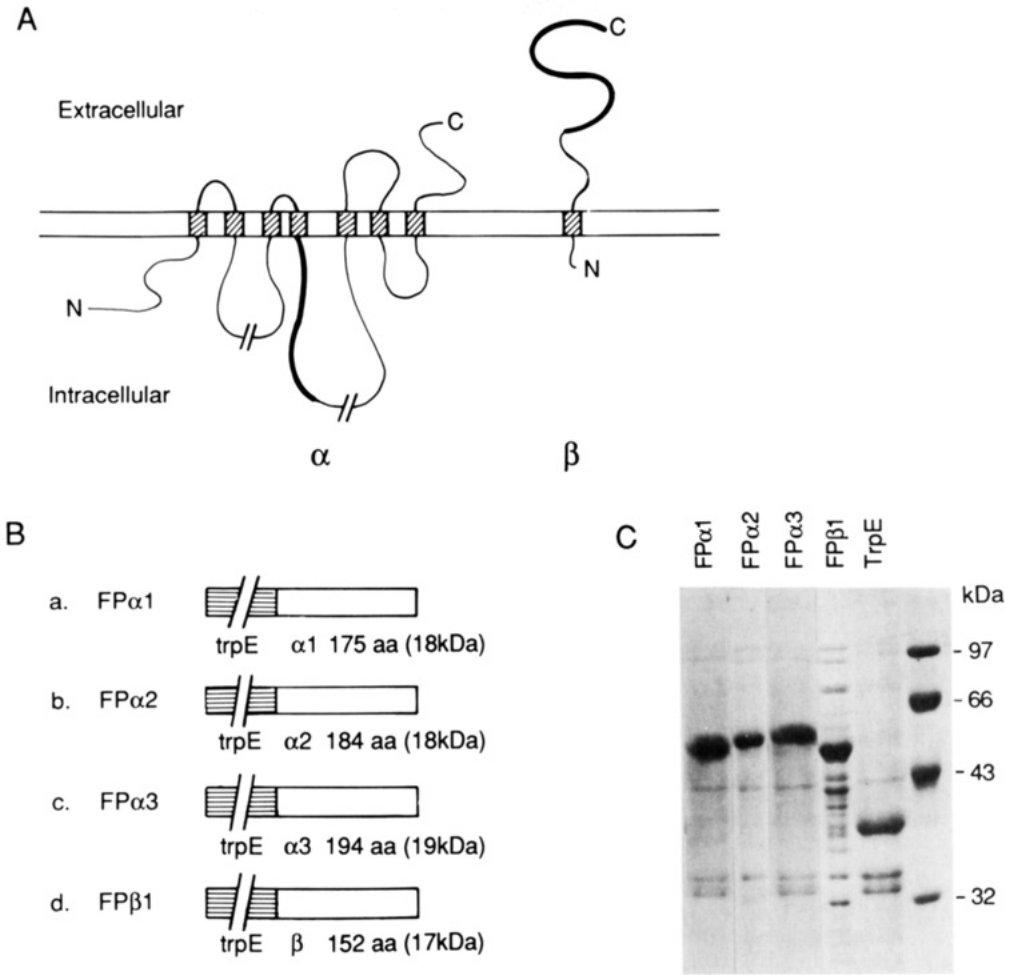

FIGURE 1: (A) Putative membrane topology of Na,K-ATPase α and β subunits based on hydropathy analysis (Herrera et al., 1987; Mercer et al., 1986). Regions of Na,K-ATPase α and β subunits encompassed by the FPα1, FPα2, or FPα3 and FPβ1 fusion proteins are highlighted. (B) Structures of the TrpE–Na,K-ATPase fusion proteins. Each fusion protein consists of the 37-kDa TrpE protein fused to (a) 175 amino acids of the α1 subunit to yield the 55-kDa fusion protein FPα1, (b) 184 amino acids of the α2 subunit to yield the 55-kDa fusion protein FPα2, (c) 194 amino acids of the α3 subunit to yield the 56-kDa fusion protein FPα3, or (d) 152 amino acids of the β subunit to yield the 54-kDa fusion protein FPβ1. (C) Coomassie blue stain of an SDS-containing 10% polyacrylamide gel showing total proteins from induced bacteria containing the unfused trpE gene and trpE–α and –β subunit fusion protein genes. Molecular mass markers are shown at the right.

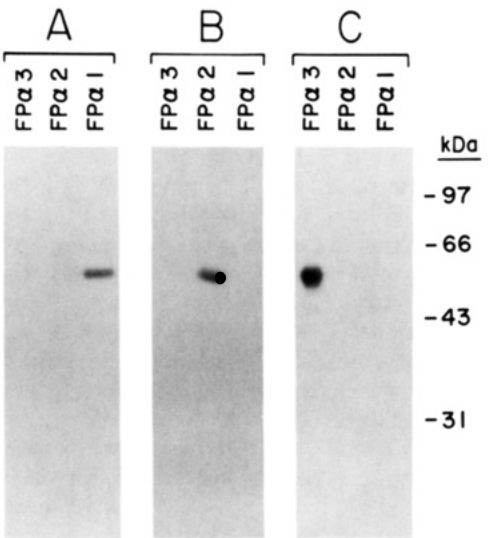

FIGURE 2: Specificity of antisera generated against TrpE–Na,K-ATPase α subunit fusion proteins. Total cellular protein from induced bacteria expressing α subunit fusion protein genes was size fractionated on SDS-containing 10% polyacrylamide gels, transferred to nitrocellulose filters, and probed with immunoabsorbed antisera generated against FPα1 (A), FPα2 (B), or FPα3 (C). Bound antibody was detected by incubating nitrocellulose filters with ^{125}I-protein A.

proteins immobilized on nitrocellulose strips (Experimental Procedures). The resulting affinity-purified antisera were tested for isoform specificity on Western blots of whole extracts of bacterial cells expressing each of the α subunit fusion proteins. As shown in Figure 2, FPα1 antiserum reacted only with α1 subunit fusion protein (panel A). FPα2 antiserum reacted with only the α2 subunit fusion protein (panel B), while FPα3 antiserum reacted only with α3 subunit fusion protein (panel C). These results establish that affinity-purified α subunit antisera are specific for their respective isotypes.

α3 Subunit Is Expressed in Rat Brain and Copurifies with Ouabain-Inhibitable Na,K-ATPase Activity. The α3 subunit of the Na,K-ATPase has not yet been identified at the protein level in vivo. A number of studies have demonstrated the presence of α3 subunit mRNA in rat brain (Emanuel et al., 1987; Orlowski & Lingrel, 1988). To determine whether α3 subunit polypeptides are also produced in this tissue, we analyzed Na,K-ATPase subunit expression in rat brain microsomes enriched for Na,K-ATPase activity. Sucrose density gradient fractions of SDS-extracted rat brain microsomes were assayed for protein concentration, ouabain-inhibitable Na,K-ATPase activity, and α3 subunit expression. As shown in Figure 3A, ouabain-inhibitable Na,K-ATPase activity sedimented in a region of the gradient distinct from the peak of

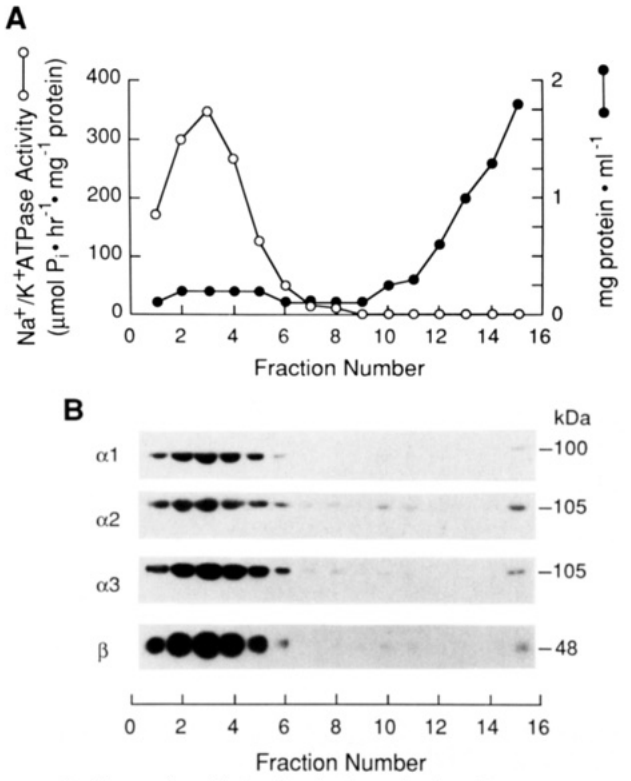

FIGURE 3: Expression of $\alpha 3$ subunits in rat brain microsomes: copurification with ouabain-inhibitable Na,K-ATPase activity. Microsomes were prepared from adult rat brain, extracted with SDS, and sedimented through a 7–30% (w/v) sucrose gradient. (A) Sucrose gradient profile of SDS-extracted brain microsomes showing protein (mg/mL) (●) and ouabain-inhibitable Na,K-ATPase activity (○). (B) Western blot analysis of sucrose gradient fractions. Aliquots from each fraction were electrophoresed through SDS-containing 7.5% polyacrylamide gels, transferred to nitrocellulose, and probed with the fusion protein derived antisera indicated at the left.

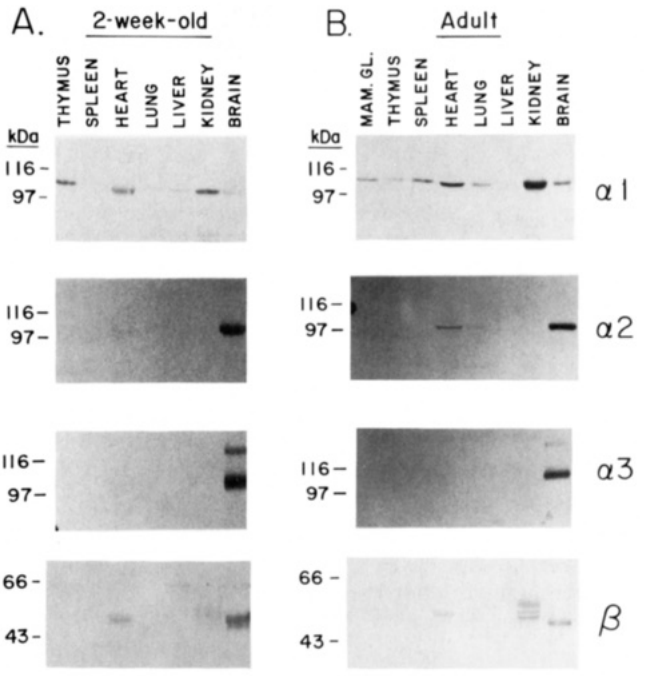

FIGURE 4: Expression of Na,K-ATPase $\alpha 1$, $\alpha 2$, $\alpha 3$, and β subunits in rat tissues. Microsomes were prepared from 2-week-old (A) and adult (B) rat tissues. Solubilized microsomal proteins (40 μg/lane) were fractionated by electrophoresis through an SDS-containing 7.5% (α subunits) or 10% (β subunit) polyacrylamide gel, transferred to a nitrocellulose filter, and probed with the fusion protein derived antisera indicated at the right. The positions of molecular mass markers are indicated on the left.

SDS-solubilized proteins. Western blot analysis of the fractions with $\alpha 3$ subunit antisera (Figure 3B) revealed that the majority of $\alpha 3$ subunits copurified with the peak of ouabain-inhibitable Na,K-ATPase activity. When duplicate blots were probed with $\alpha 1$, $\alpha 2$, and β subunit antisera, each of these Na,K-ATPase subunits cosedimented with the peak of enzyme activity. These results demonstrate that $\alpha 3$ subunit polypeptides are expressed in rat brain and are present in a membrane fraction enriched in Na,K-ATPase activity.

Tissue-Specific Expression of Na,K-ATPase α Subunit Polypeptides. To analyze expression of Na,K-ATPase α subunit polypeptides, antisera specific for each of the α subunit isoforms were used to probe Western blots containing rat tissue microsomal fractions. The results are shown in Figure 4. The $\alpha 1$ subunit antisera reacted with a polypeptide species of ~100 kDa, whereas $\alpha 2$ and $\alpha 3$ subunit antisera reacted with polypeptides of ~105 kDa. The $\alpha 3$ antiserum also reacted with a polypeptide of ~130 kDa, which probably represents nonspecific binding since antiserum produced from a separate rabbit (immunized with FP$\alpha 3$) failed to immunoreact with this polypeptide. In 2-week-old rats (panel A), $\alpha 1$ subunit polypeptides were most abundant in kidney, heart, and thymus, less abundant in brain, liver, and lung, and least abundant in spleen. In contrast, $\alpha 2$ subunit polypeptides were detected at high levels in brain and at trace levels in heart. $\alpha 3$ subunit polypeptides were predominant in brain with very low levels present in heart (visualized on original blot).

In the adult (Figure 4B), $\alpha 1$ subunit polypeptides were most abundant in kidney. Moderate levels of the $\alpha 1$ subunit were detected in heart, and lower levels were present in brain, lung, spleen, and lactating mammary gland, while very low levels were detected in liver and thymus. $\alpha 2$ subunit polypeptides were detected at highest abundance in brain, at moderate levels in heart, and at low abundance in lung. Expression of the $\alpha 3$ subunit was restricted entirely to brain. These results demonstrate that multiple forms of the Na,K-ATPase α subunit are expressed in a tissue-specific and developmentally regulated fashion.

Tissue-Specific Expression of the Na,K-ATPase β Subunit. The pattern of expression of the Na,K-ATPase β subunit is shown in Figure 4 (β panel). In 2-week-old rats (panel A), β subunit polypeptides were most abundant in brain and heart and moderately abundant in kidney. Polypeptides of ~48 and 50 kDa in size were present in brain. A broad band ranging from ~50 to 60 kDa was detected in kidney. Interestingly, no β subunit polypeptides could be detected in liver, lung, spleen, or thymus. A 60-kDa band in liver did react with β subunit antiserum. However, this probably results from nonspecific binding since β subunit antiserum produced from a separate rabbit failed to immunoreact with this polypeptide.

In the adult (panel B), β subunit polypeptides were most abundant in kidney, moderately abundant in brain, and less abundant in heart. No β subunit polypeptides were detected in liver, lung, spleen, thymus, and mammary gland. Polypeptides of ~52 and 54 kDa in size were detected in heart, while polypeptides of ~48 and 50 kDa were present in brain. At least five immunoreactive bands, ranging from ~50 to 60 kDa, were detected in kidney. These results indicate that in several rat tissue membranes the β subunit exhibits a pattern of expression distinct from that of any of the α subunits.

Discussion

We have used cloned fusion proteins as antigens to generate antisera specific for the three α subunit isoforms and one β subunit of the rat Na,K-ATPase. Utilizing these antisera, we

show for the first time that the Na,K-ATPase $\alpha 3$ subunit gene produces a polypeptide that is present in rat brain membranes. Second, we demonstrate that the protein products of each Na,K-ATPase subunit gene exhibit a distinct tissue-specific pattern of expression. Third, we provide evidence for the apparent absence of β subunits (reactive with FPβ1-derived antisera) in several rat tissue membranes. The distinct tissue distributions of Na,K-ATPase subunits may reflect important differences in the functions of individual ATPase isoforms.

The Na,K-ATPase plays an important role in the functioning of the brain. The enzyme is found in high concentrations in neurons, where it maintains the sodium and potassium gradients that are essential for nerve impulse generation (Thomas, 1972). The Na,K-ATPase is also present in glial cells, which are involved in the buffering of extracellular potassium following nerve activity (Hertz, 1977). A number of studies have demonstrated the presence of $\alpha 1$ and $\alpha 2$ subunit polypeptides in rat brain (Sweadner, 1979; Schmitt & McDonough, 1986). Our results show that the $\alpha 3$ subunit isoform is also expressed in brain. The presence of $\alpha 3$ subunits in a membrane fraction enriched for Na,K-ATPase activity is consistent with the view that the $\alpha 3$ subunit is a functional component of the brain enzyme. It will clearly be of interest to determine whether differential subcellular localization of α subunit isoforms occurs in the brain and other tissues.

Recent studies have demonstrated substantial differences in the tissue and developmental specificity of expression of mRNAs encoding three rat Na,K-ATPase α subunit isoforms (Emanuel et al., 1987; Orlowski & Lingrel, 1988). Interestingly, we find marked differences in the the levels of expression of α subunit mRNAs and polypeptides in several rat tissues. For example, in kidney and liver, no $\alpha 2$ and $\alpha 3$ subunits were detected despite the presence of $\alpha 2$ and $\alpha 3$ subunit transcripts in these tissues (Emanuel et al., 1987). The apparent differences between $\alpha 2$ and $\alpha 3$ subunit mRNA and protein levels could have important implications with regard to translational regulation of $\alpha 2$ and $\alpha 3$ subunit transcripts. Alternatively, this discordance could reflect differences in the sensitivity between Northern and Western blotting analysis or variations in mRNA expression levels from rat to rat. Current efforts are directed at resolving this issue.

Differences in the distribution of α and β subunits in several rat tissue membranes raise interesting questions regarding the overall control of Na,K-ATPase expression. It is generally accepted that the molar ratio of α/β subunits in the sodium pump holoenzyme is 1:1 (Cantley, 1981). In rat liver, spleen, thymus, and lactating mammary gland, we were unable to detect expression of any β subunit polypeptides despite the presence of $\alpha 1$ subunits in these tissues. These results suggest that the β subunit may not be essential for enzyme activity. However, if relatively equal levels of expression of α and β subunits are required for Na,K-ATPase activity, then it is possible that the β subunit may be a member of a multigene family. The identification of a putative $\beta 2$ subunit isoform of the rat Na,K-ATPase β subunit (Martin-Vasallo et al., 1989) is consistent with this hypothesis. It will be of interest to determine whether the pattern of expression of the $\beta 2$ isoform is different from the β subunits characterized in this study.

At present, we have little insight into the functional significance for multiple forms of Na,K-ATPase α and β subunits. Differences in the intracellular distribution of the isoforms hint at underlying functional differences. A further issue that should be raised in this context is the possibility that alternate combinations of α and β subunits produce Na,K-ATPases with differing physiological properties. The availability of antisera specific for each subunit isoform should permit identification of the α and β subunits present within different Na,K-ATPase holoenzymes.

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