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Ontogeny of basolateral membrane sodium-hydrogen exchange (NHE) activity and mRNA expression of NHE-1 and NHE-4 in rat kidney and jejunum

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Abstract

Na^+/H^+ exchange (NHE) activity varies with ontogenic state in rat intestinal basolateral membrane vesicles (BLMV). The current investigation sought to determine if these observations are due to differential expression of BLM NHE isoforms, NHE-1 and NHE-4. In rat kidney, BLMV sodium uptake levels were similar in 2, 3 and 6 week rats (13.28 ± 0.68, 14.03 ± 0.84, and 11.71 ± 0.66 nmol Na^+/mg protein/30 s, respectively), and lower in adults (5.53 ± 0.24) ($n = 4$; $p < 0.001$ between 2 week rats and adults, and between 3 week rats and adults; $p < 0.01$ between 6 week rats and adults). In rat jejunum, BLMV uptake was highest in adults (13.07 ± 0.86 nmol Na^+/mg protein/30 s), and decreased in 6, 3, and 2 week rats (4.48 ± 0.75, 2.94 ± 0.68, and 1.59 ± 0.58, respectively) ($n = 4$; $p < 0.001$ between all groups and adults). Control immunoblot experiments with NHE-3 antiserum showed that BLMV preps were not contaminated with significant amounts of this brush-border membrane specific protein. Northern blots with isoform-specific probes showed highest renal NHE-1 hybridization intensities in 2 and 3 week rats (11.00 ± 0.25 and 12.07 ± 0.16 phosphorimage units, respectively), and lower intensities in 6 week and adult animals (4.30 ± 0.95, and 4.40 ± 1.40, respectively) ($n = 4$; $p < 0.01$ between 2 week animals and 6 week and adult animals, and between 3 week animals and 6 week and adult animals). NHE-1 probes in the intestine showed no hybridization intensity differences between groups: 2 week-7.09 ± 1.10, 3 week-5.39 ± 0.56, 6 week-8.24 ± 1.57, and adult-8.99 ± 2.20 ($n = 3$). NHE-4 specific probes in the kidney showed hybridization intensity levels of 9.22 ± 0.35 in 2 week animals, 12.12 ± 1.26 in 3 week animals, 5.63 ± 0.81 in 6 week animals, and 3.52 ± 0.57 in adults ($n = 4$; $p < 0.05$ between 2 week and adults; $p < 0.01$ between 3 week and 6 week animals, and between 3 week and adults). No NHE-4 message was detected in rat jejunum by Northern blot analysis or by reverse transcriptase-PCR. These results suggest that ontogenic NHE activity at the jejunal BLM is not related to differential expression of NHE-1, while NHE activity at the renal BLM may in part be related to differential ontogenic expression of NHE-1 and NHE-4. © 1998 Elsevier Science B.V.

Keywords: Sodium-hydrogen exchange; Jejunum; Kidney; Ontogeny; (Rat)

1. Introduction

The sodium-hydrogen exchangers (NHE) are plasma membrane bound transport proteins that function to exchange cytoplasmic H^+ for extracellular

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Na^+. The first NHE identified and cloned at the molecular level was called NHE isoform-1 (NHE-1) [1]. Subsequently, several other isoforms of NHEs have been identified [2,3,5–7], and these have been designated NHE- 2, 3, and 4. NHE-2 and NHE-3 have been localized to the brush-border in intestinal and renal epithelia, and are thought to play important roles in sodium absorption. Conversely, NHE-1 and NHE-4 have been localized to the renal and intestinal basolateral membrane (BLM), and these isoforms are thought to be involved in cell volume and pH regulation and cellular proliferation. Additionally, the BLM NHE isoforms may play a role in hypertension and other human disorders related to problems with cell proliferation. The potential link between the BLM NHEs and these human diseases in man makes their investigation clinically relevant.

NHE-1 is expressed in most tissues and has been cloned from several species [1,3,4,8,9]. This isoform is involved in cell volume and intracellular pH regulation [1]. NHE-1 mRNA is expressed in nearly all mammalian cells [4], and the NHE-1 protein has been localized to the BLM in rabbit ileum [4], LLC-PK_1 [9], Caco-2 cell lines [10], and rabbit kidney epithelium [11]. Subsequent to the original cloning, tissue expression and kinetic characterization of NHE-2 from rat intestine [2,12,13], this isoform was cloned from several species [5,7,14]. NHE-2 is likely involved in intestinal transepithelial Na^+ absorption, and additionally may play a role in cell volume regulation in renal medullary collecting duct cells [15]. NHE-2 mRNA was detected in several mammalian tissues [2], and NHE-2 protein has been localized to the brush-border membrane (BBM) in human and rabbit intestinal and renal epithelia [15,16]. NHE-3 has been cloned from several species [3,6,17], and this isoform is also likely involved in intestinal transepithelial sodium absorption. NHE-3 message was detected in rabbit kidney, intestine and stomach [3,6], and NHE-3 protein has been localized to the BBM in rabbit and human ileum [15], and rabbit renal tubules [16]. NHE-4 has been cloned from the rat [3]. The NHE-4 message is expressed in several rat tissues including stomach, intestine, and kidney. NHE-4 has an unknown function, and the NHE-4 protein was recently shown to be expressed on the BLM in the rabbit renal medulla [18]. It, therefore, appears that 2 of the known NHE isoforms are expressed in mammalian intestinal and renal BBM (NHE-2 and NHE-3), and the other 2 isoforms are expressed on the BLM (NHE-1 and NHE-4).

The NHEs have been extensively investigated at the functional level for many years. With the recent molecular identification of several isoforms of these transport proteins, the NHEs may now be studied at the molecular level. Previous studies by group have been designed to investigate the ontogenic expression of the BBM NHE isoforms, but no previous work has been done on the ontogenic expression of the BLM NHE isoforms at the molecular level. However, it has been previously documented that NHE activity varies with ontogenic state in rat intestinal BLM vesicles (BLMV) [20]. Similar investigations have not been previously performed in mammalian renal BLMV. Therefore, the current investigation was undertaken to determine if these variable levels of NHE activity during rat ontogeny are due to differential expression of one or more of the known BLM NHE isoforms. Since NHE-1 and NHE-4 have been localized to the BLM, the current investigation will focus on these two isoforms.

2. Methods

2.1. Animals

Sprague Dawley rats were used in groups of at least 4 animals for all experiments. Male rats of the following ages were used for all studies: 2 week (sucklings) at 14 days of age, 3 week (weanlings) at 21 days of age, 6 week (adolescents) at 42–45 days of age, and adult at 4–6 months of age. Animals were maintained with food and water supplied ad libitum. Suckling and weanling animals were in the presence of the maternal rat. Weanling rats were presumably feeding on milk and rat chow. Animals were subjected to CO_2 narcosis, and sacrificed by cervical dislocation.

2.2. Chemicals and reagents

Poly $(A)^+$ RNA was isolated utilizing the Fast Track kit from Invitrogen (La Jolla, CA). [^{22}Na] (100–2000 Ci [3.70–74.0 Tbq]/g) for uptake studies, and α-[^{32}P]-dCTP (3000 Ci/mmol) for Northern blot analyses were purchased from New England Nuclear

(Boston, MA). Radioactive probes for Northern blot analyses were generated by the RediPrime system from Amersham Life Science (Piscataway, NJ). Nitrocellulose membranes (nitroplus) were from Micron Separations (Westboro, MA). NHE-1 and NHE-4 cDNA containing plasmids were kindly provided by Dr. Gary Shull (University of Cincinnati, OH) [3]. TMA-gluconate was made by titrating solutions of TMA-hydroxide with gluconic acid. Protein was quantitated by a Bradford assay utilizing the BioRad Protein Assay reagent (Hercules, CA). PCR products were subcloned with the PGEM-T subcloning system (Promega Corp.; Madison, WI). DNA was purified from agarose gels utilizing the GeneClean kit from BIO101 (Vista, CA). Lipofectamine was purchased from Gibco-BRL (Bethesda, MD). DMEM and all other cell culture reagents were from Irvine Scientific (Irvine, CA). All other chemicals and reagents were purchased from Fisher Biotechnology (Pittsburgh, PA), or Sigma (St. Louis, MO).

2.3. Basolateral membrane vesicle (BLMV) isolation

BLMV were purified utilizing a modified centrifugation technique followed by separation on a percoll gradient essentially as previously described [20]. For renal BLMV preps, whole kidney tissue was harvested and placed in 10 ml buffer A (250 mM sucrose, 2 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF), and 2 mM HEPES/Tris, pH 7.4), and homogenized in a glass homogenizer. The sample was spun for 10 min at $1000 \times g$, and the supernatant was recovered and spun at $22{,}000 \times g$ for 15 min. The resulting pellet was suspended in 8.5 ml buffer A, homogenized, and mixed with 1.5 ml 100% percoll and spun at $48{,}000 \times g$ for 75 min. The uppermost band was aspirated and diluted with 5 volumes of buffer B (85 mM sucrose, 85 mM KCl, 0.1 mM PMSF and 2 mM HEPES/Tris, pH 7.4). The sample was spun at $48{,}000 \times g$ for 20 min, and the pellet was suspended in buffer C (100 mM sucrose, 100 mM KCl, 0.1 mM PMSF and 2 mM HEPES/Tris, pH 7.4). The sample was split into 2 tubes and spun again at $48{,}000 \times g$ for 20 min. The final BLMV pellets were resuspended in either pre-incubation buffer for no pH gradient condition ($pH_i/pH_o = 7.5/7.5$) (100 mM TMA-gluconate, 0.1 mM PMSF, 85 mM HEPES, 45 mM Tris/HCl (pH 7.5)), or pre-incubation buffer with outwardly directed pH gradient ($pH_i/pH_o = 5.2/7.5$) (100 mM TMA-gluconate, 0.1 mM PMSF, 90 mM MES, 40 mM HEPES/Tris (pH 5.2)).

For intestinal BLMV preparations a similar technique was utilized. Jejunal segments were excised from animals, flushed with saline solution, and filled with 37°C citrate buffer (15 mM KCl, 96 mM NaCl, 8 mM KH_2PO_4, 5.6 mM Na_2PO_4, 27 mM NaCitrate, 2 mM dithiothreitol (DTT), 0.1 mM PMSF, pH 7.4) for 15 min at 37°C. The contents were emptied, and the segments were filled with ice-cold buffer 1 (250 mM mannitol, 0.1 mM PMSF, 12 mM HEPES, 2 mM Tris, pH 7.4) and palpated for 5 min. The intestinal segment was then drained into 30 ml ice-cold buffer 1 and spun at $200 \times g$ for 5 min. The resulting pellets were then homogenized in 60 ml ice-cold buffer 1, and spun for 20 min at $2500 \times g$. The supernatant was collected and spun at $22{,}000 \times g$ for 25 min. The pellet was suspended in 8.5 ml buffer 1, homogenized with a glass homogenizer, mixed with 1.5 ml 100% percoll, and spun at $48{,}000 \times g$ for 75 min. The upper band was aspirated, resuspended in buffer 2 (100 mM mannitol, 100 mM KCl, 0.1 mM PMSF, 12 mM HEPES, 2 mM Tris, pH 7.4), and spun at $48{,}000 \times g$ for 20 min. The pellet was resuspended in 3 ml buffer 3 (280 mM mannitol, 0.1 mM PMSF, 20 mM HEPES, 2 mM Tris, pH 7.4), split into 2 tubes and spun at $48{,}000 \times g$ for 20 min. The resulting 2 pellets were resuspended in pre-incubation buffer for pH gradient or no pH gradient conditions as described above. Enrichments of both renal and intestinal preps for BLM was assessed by measuring Na^+/K^+-ATPase activity in crude homogenates and in final pellets as previously described [20].

2.4. Uptake analysis of renal and intestinal BLMV

Uptake of radiolabeled sodium was measured by a rapid filtration technique as previously described [20–23]. All incubations were done at 25°C. Previous experimentation had determined that the linear part of uptake curve (initial rate) extended out to at least 120 s in suckling, weanling and adolescent rats [19]. Membrane pellets were suspended in pre-incubation buffer for pH gradient or no pH gradient conditions 1 h prior to transport measurements (see Section 2.3). Transport was initiated by adding 20 μl of the final

membrane solution to 80 μl incubation solution (same as pre-incubation buffer (pH 7.5), with the addition of 31.25 μCi [^{22}Na]/10 ml plus 1 mM NaCl). The reactions were stopped after 30 s by the addition of 2 ml ice-cold stop solution (185 mM potassium-gluconate, 10 mM Tris, 16 mM HEPES, 0.1 mM amiloride). The vesicles were immediately collected on a cellulose nitrate filter (0.45 μm pore size) and kept under suction while they were washed with 5 ml ice-cold stop solution. The amount of radioactive substrate remaining on the filter was determined in a Beckman liquid scintillation counter, with ReadySafe (Beckman; Fullerton, CA) as the liquid scintillant. Radioactivity remaining in the filters after pipetting incubation medium into the radioactive substrate in the absence of vesicles was used as background, and was considered into all calculations. Uptake values were determined by subtracting the uptake levels for no pH gradient condition ($pH_i/pH_o = 7.5/7.5$) from those for outwardly directed pH gradient ($pH_i/pH_o = 5.2/7.5$). All values are expressed as nmol of sodium uptake per mg of vesicle protein per 30 s. Graphical representation is expressed as mean ± S.E.M. of four uptake assays for each age group and tissue.

2.5. Northern blot analysis of rat kidney and jejunum

Poly $(A)^+$ RNA was isolated from the whole kidney and jejunal mucosa of at least four rats per group utilizing a commercially available kit. This method uses a tissue lysis buffer containing RNAse and protein degraders, and oligo D(T)-cellulose affinity chromatography. Northern blots were carried out as previously described using 5 μg poly $(A)^+$ RNA per lane [24,25]. Denaturing agarose (1%) gel electrophoresis was followed by blotting overnight onto a nitrocellulose membrane. Probes were generated from a DNA fragment of NHE-1 cDNA encompassing 1148–3684 bp, and from an NHE-4 cDNA PCR fragment encompassing 1951–2326 bp. Both of these probes were designed based upon their lack of nucleotide sequence similarity with any of the other NHE isoforms, and they were used for Northern blot analyses based upon their lack of cross-hybridization with the other NHE isoforms in slot blot experiments [19]. 1B15 (encoding rat cyclophilin) [26] cDNA was used to generate constitutive probes. All probes were made by random prime labeling with α-[^{32}P]-dCTP, and 1×10^6 counts per min (cpm) were used per ml of hybridization solution. 1B15 has been extensively used by our group as a constitutively expressed gene for comparison in a variety of experiments [27,28]. The blots were processed as extensively previously described [27]. After hybridization at 65°C with a dextran sulfate containing hybridization buffer, high stringency washes were performed at 65°C with 0.1 times SSC, 0.1% SDS, and blots were placed to a phosphorimaging screen or film. Blots were stripped and subsequently reprobed with 1B15 specific probes as previously described [27]. Quantitation of hybridization signals was done by phosphorimage analysis utilizing volume integration (GS 525 Molecular Imager; BioRad; Hercules, CA). Utilizing phosphorimaging technology allows precise determination of hybridization band intensities, and it also makes it possible to determine if signals are within the linear range of the imaging screens. Experiments were performed in triplicate or quadruplicate with poly $(A)^+$ RNA samples from different groups of animals, and hybridization intensities were averaged from the multiple experiments. Hybridization intensities were normalized for 1B15 levels on the same blot.

2.6. RT-PCR analysis of rat jejunum with NHE-2 and NHE-4 primers

In order to confirm the absence of NHE-4 in rat jejunum, the RT-PCR technique was utilized with NHE-2 and NHE-4 specific primer pairs as previously described [2,24]. Poly $(A)^+$ RNA was isolated from 3 week rat jejunal mucosal scrapings, and converted to cDNA by MMLV reverse transcriptase. RT-PCR reactions were run with the NHE-2 or NHE-4 specific primer pairs, and PCR products were fractionated by 1% agarose gel electrophoresis. The following set of NHE-2 primers was used: left primer-5′CAGCGCACATTGTCCTACAA3′ at 2041 bp (based upon the sequence of the NHE-2 cloned in our lab [2]), and right primer-5′TGTC-CGAGTCGCTGCTATTA3′ at 2293 bp. The following 2 sets of NHE-4 primers were utilized: left primer 1-5′GATACTCCTCGCATCTCTTG3′ at 422 bp, right primer 1-5′AGGATGGAGCCAATGTTCTC3′ at 670 bp, left primer 2-5′CCGAAGACGTGGAATC-CATG3′ at 1951 bp, and right 2-5′AGCCTTG-

GCTGTGTCTTCTG3′ at 2306 bp. Positive control reactions were run with NHE-2 or NHE-4 containing plasmid DNA and negative controls were identical reactions with no DNA.

2.7. NHE-3 antiserum production and characterization

In an effort to raise antibodies against the BBM specific NHE-3 isoform, we produced a recombinant fusion protein of NHE-3. NHE-3 cDNA was amplified between 2183–2581 bp (a region with no amino acid or nucleotide sequence homology to other NHE isoforms). The resulting DNA fragment spanned amino acids 699–831 from the C-terminal portion of the protein. The PCR product was subcloned into pBlueBacHis vector (Invitrogen; La Jolla, CA), to create pBlueBacHis/NHE-3. This construct was co-transfected with Bac-N-Blue baculovirus DNA into Sf9 insect cells by cationic liposome mediated transfection. This procedure results in recombinant viral DNA which can infect and replicate in insect cells. Several putative positive clones were analyzed by RT-PCR and immunoblot analysis, and from these experiments a positive baculovirus clone was selected that showed highest expression of NHE-3 fusion protein. Large scale protein purifications utilizing high-affinity metal chromatography (1 l cell culture volume) led to the recovery of between 1.2–1.5 mg of recombinant fusion. The recovered recombinant protein was intradermally injected into 2 rabbits for production of polyclonal antibodies specific for the C-terminal epitopes of the NHE-3 protein.

In order to characterize the NHE-3 antiserum, we transfected PS120 cells with NHE-2 and NHE-3 cDNAs as follows: NHE-2 cDNA was amplified by PCR with the following primer pair: left primer at bp 147-5′TACACTGGCGTCCTCTGCTG3′, and right primer at bp 2678-5′AAGTCACAACCATGCTTGCC3′. NHE-3 cDNA was similarly amplified by PCR utilizing the following primer pair: left primer at bp 33-5′AAGAGCGCACGAGGTACCAC3′, and right primer at bp 2717-5′AGTCGAAGTGCGCTCAGGTG-3′. These primers allowed amplification of the entire open reading frame of NHE-2 and NHE-3. PCR conditions for both amplifications were as follows: 94°C for 4 min (step 1), 94°C for 1 min (step 2), 58° for 1 min (step 3), 72°C for 2 min (step 4) and cycled between step 2 and step 4 (30 times) followed by 72°C for 10 min. PCR products were subcloned into the pTarget vector (Promega Corp.; Madison, WI) which is a eukaryotic expression vector that is designed for direct subcloning of PCR products. Ampicillin resistant bacterial colonies were screened by PCR for the presence of the NHE-2 or NHE-3 cDNA insert. PCR product/pTarget DNA constructs were sequenced by the dideoxy chain termination method.

PS120 cells were maintained as previously described [2]. Cells were transfected with NHE-2 or NHE-3 expression constructs by a liposome mediated transfection procedure with lipofectamine essentially by the manufacturer's protocol. After transfections, cells containing expression plasmids were selected for by exposure to 800 μg/ml G418 antibiotic for 7–10 days. In order to further select for cells transfected with NHE-2 or NHE-3, cells were acid loaded as previously described [2]. Briefly, cells were incubated in acid medium (50 mM NH_4Cl, 72 mM choline, 4.9 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 20 mM HEPES, pH 7.5) for 1 hr at 37°C. Cells were rinsed twice with choline solution (122 mM choline, 4.9 mM KCl, 1 mM $CaCl_2$, 2.5 mM Mg_2SO_4, 20 mM HEPES, pH 7.0), and incubated in NaCl solution (122 mM NaCl, 4.9 mM KCl, 1 mM $CaCl_2$, 2.5 mM Mg_2SO_4, 20 mM HEPES, pH 7.5) for 1 h at 37°C. The cells were then rinsed with medium plus antibiotics and incubated at 37°C for 48 h. The acid selection protocol was repeated 2–3 additional times. Then, cells were scraped or trypsinized from dishes and used for poly $(A)^+$ RNA purification for reverse transcriptase-PCR or for crude membrane protein purification for immunoblot analysis with NHE-3 antiserum.

In order to assay for NHE-2 or NHE-3 mRNA expression in transfected cells, the RT-PCR technique was performed as previously described [2,24]. When cells reached confluency after the final acid selection, cells were trypsinized from the dishes and poly $(A)^+$ RNA was isolated with a commercially available kit (MicroFastTrack system; Invitrogen; La Jolla, CA). mRNA was converted into cDNA by MMLV reverse transcriptase. RT-PCR reactions were run with the NHE-2 or NHE-3 specific primer pairs, and PCR products were fractionated by 1% agarose gel electrophoresis.

For immunoblot analyses, crude membranes were

prepared from transfected and untransfected PS120 cells that were grown in 100 mm dishes. Medium was decanted and the cells were washed with ice-cold PBS (pH 7.5). Then, 3 ml extraction buffer was added to cell monolayers (10 mM Tris (pH 7.5), 1 mM EDTA, 0.1 mM PMSF, 1 mM iodoacetamide, 1 mM *o*-phenanthroline), the cells were scraped from dishes and transferred to a centrifuge tube. The cells were next homogenized with a 26 gauge needle and spun at $1000 \times g$ for 30 min at 4°C. The supernatant was decanted to a new tube and spun at $40{,}000 \times g$ for 30 min at 4°C. The resulting pellet was resuspended in TE buffer. The solution was again homogenized with a 26 gauge needle and protein assays were performed as previously described. Proteins were solubilized in Laemmli buffer plus βME, boiled for 5 min, fractionated by SDS/PAGE and transferred to nitrocellulose membranes as previously described [23,25]. Membranes were reacted with NHE-3 antiserum at 1:4000 dilution.

In order to further characterize NHE-3 antiserum, intestinal and renal brush-border membrane proteins were purified by a $MgCl_2$ precipitation method as previously described [12,25]. 10–20 μg of protein was placed in a 1:1 dilution of Laemmli solubilization buffer plus 2 mM β-mercaptoethanol (βME), boiled for 5 min and placed on ice. Protein samples were fractionated by 8% SDS polyacrylamide gel electrophoresis (SDS/PAGE), and transferred onto nitrocellulose membranes. Blots were processed with the Renaissance chemiluminescent system (NEN/Dupont; Boston, MA) with 1:4000 dilutions of NHE-3 specific antisera. Additionally, some blots were reacted with pre-immune serum and serum that was pretreated with antigenic fusion protein (0.4 mg/ml at 10°C for 16 h) at 1:4000 dilution. Membranes were stripped and subsequently reacted with β-actin antiserum at 1:4000 dilution. Some blots were reacted with monoclonal mouse anti-Na^+/K^+-ATPase antiserum (Affinity Bioreagents; Golden, CO) at 1:2000 dilution. NHE-3 bands were visualized by phosphorimage analysis utilizing chemiluminescent imaging screens (BioRad; Hercules, CA).

2.8. Computer analysis of NHE cDNA sequences

DNA sequences were analyzed for restriction sites by the program RESTRI and sequence alignments were done with the program NALIGN within the PC/GENE nucleic acid and protein sequence analysis software system (A. Bairoch; University of Geneva, Switzerland; Intelligenetics).

2.9. Statistical analysis of results

Data from uptake studies and Northern blot analyses were analyzed for statistical significance by ANOVA, utilizing the GraphPad InStat program (GraphPad software; San Diego, CA), and are presented as mean ± S.E.M. Additionally, post tests were performed by the method of Bonferroni to look at statistical differences between individual groups.

3. Results

3.1. Uptake analysis of renal and intestinal BLMV

BLMV were prepared from kidney and jejunum of groups of rats at the various ages. Enrichment of renal and jejunal membrane preparations for Na^+/K^+-ATPase activity was 8–10-fold as compared to crude tissue homogenate, which is consistent with previous observations [20]. Uptake of radiolabeled sodium was assayed under outwardly directed pH gradient and no pH gradient conditions (which were subtracted from the numbers for pH gradient conditions). In rat kidney, BLMV sodium uptake levels were similar in 2, 3, and 6 week rats (13.28 ± 0.68, 14.03 ± 0.84 and 11.71 ± 0.66 nmol Na^+/mg protein/30 s, respectively), and lower in adults (5.53 ± 0.24) ($n = 4$; $p < 0.001$ between 2 week rats and adults, and between 3 week rats and adults; $p < 0.01$ between 6 week rats and adults). No differences were apparent in values obtained from 2, 3, and 6 week animals. In rat jejunum, BLMV uptake was highest in adults (13.07 ± 0.86 nmol Na^+/mg protein/30 s), and decreased in 6, 3 and 2 week rats (4.48 ± 0.75, 2.94 ± 0.68, and 1.59 ± 0.58, respectively) ($n = 4$; $p < 0.001$ between all groups and adults). No differences were apparent in values generated from 2, 3, and 6 week animals. Data is expressed graphically in Fig. 1.

3.2. Northern blot analysis of rat kidney and jejunum

Poly $(A)^+$ RNA was isolated from jejunum and kidney of groups of rats at desired ages, fractionated

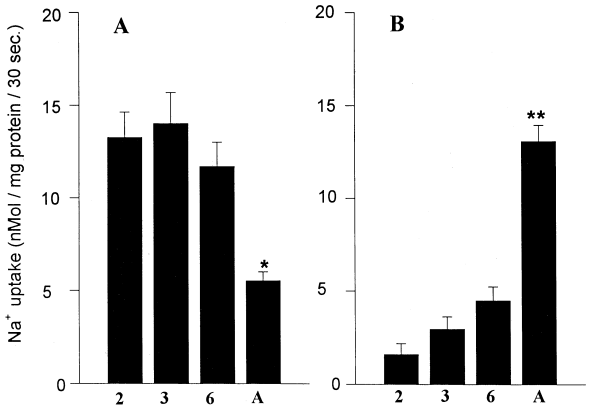

Fig. 1. pH-dependent sodium uptake by rat renal and jejunal BLMV. BLMV were prepared from groups of rats at the desired age, and were assayed for pH-dependent uptake of radiolabeled sodium. Panel A depicts renal BLMV uptake from the 4 age group rats, and panel B depicts jejunal BLMV uptake. Numbers on the left of each panel represent sodium uptake in nmol/mg protein/30 s. Standard error bars are present for each column. Two is 2 week rats, 3 is 3 week rats, 6 is 6 week rats, and A is adult rats ($n = 4$ for all groups). $^{*}p < 0.001$ between 2 week rats and adults, and between 3 week rats and adults; $p < 0.01$ between 6 week rats and adults, $^{**}p < 0.001$ between all groups and adults.

on agarose gels and transferred to nitrocellulose. Blots were reacted with radiolabeled probes that were specific for NHE-1 or NHE-4. Blots were additionally reacted with 1B15 specific probes as a constitutively expressed message (Fig. 2). Renal NHE-1 hybridization intensities were highest in 2 and 3 week rats (11.00 ± 0.25 and 12.07 ± 0.16 phosphorimage units, respectively), and lower in 6 week and adult animals (4.30 ± 0.95, and 4.40 ± 1.40, respectively) ($n = 4$; $p < 0.01$ between 2 week and 6 week, 2 week and adult, 3 week and 6 week, and 3 week and adult). Data for renal NHE-1 hybridizations is presented graphically in Fig. 3. NHE-1 probes in the intestine showed no hybridization intensity differences between groups: 2 week-7.09 ± 1.10, 3 week-5.39 ± 0.56, 6 week-8.24 ± 1.57, and adult-8.99 ± 2.20 ($n = 3$).

NHE-4 specific probes in the kidney showed hybridization intensity levels of 9.22 ± 0.35 in 2 week animals, 12.12 ± 1.26 in 3 week animals, 5.63 ± 0.81 in 6 week animals, and 3.52 ± 0.57 in adults ($n = 4$; $p < 0.05$ between 2 week and adult; $p < 0.01$ between 3 week and 6 week, and between 3 week and adult). No differences were noted between 2 and 3

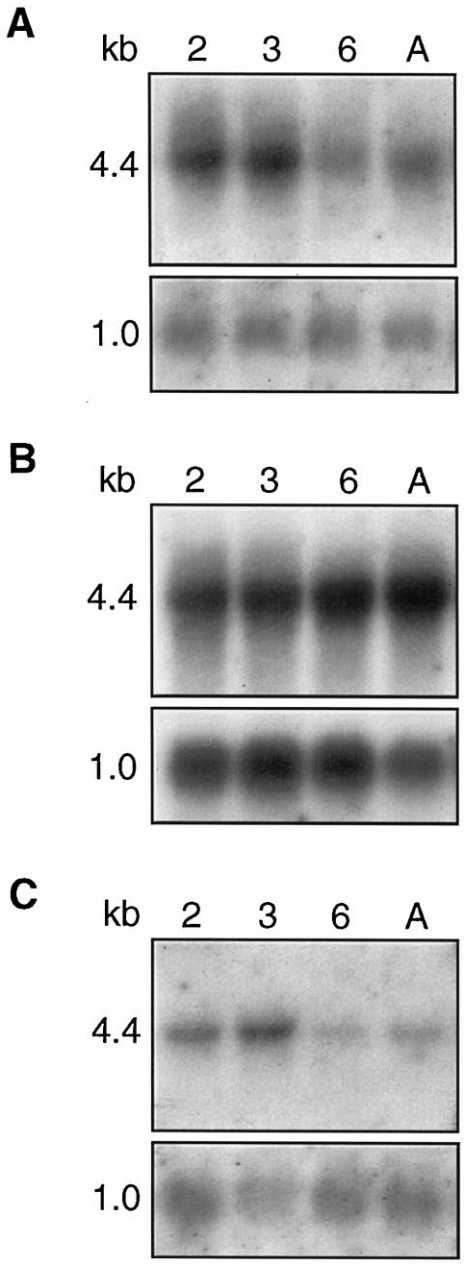

Fig. 2. Northern blot analysis of rat kidney and jejunum with NHE-1 and NHE-4 isoform specific probes. Poly $(A)^+$ RNA was isolated from jejunum and kidney of groups of desired age rats, fractionated by denaturing agarose gel electrophoresis (5 μg/lane), and transferred onto nitrocellulose membranes. Panel A depicts NHE-1 probes hybridized to rat kidney RNA, panel B depicts NHE-1 probes hybridized to rat jejunal RNA, and panel C depicts specific NHE-4 probes hybridized to rat renal RNA. In all panels, the hybridization signal at 4.4 kb represents the NHE message, and the hybridization signal to 1.0 kb represents 1B15 message. Two is 2 week rats, 3 is 3 week rats, 6 is week rats, A is adult rats, and kb is kilobase pairs. Data for panels A and C are presented graphically in Fig. 3.

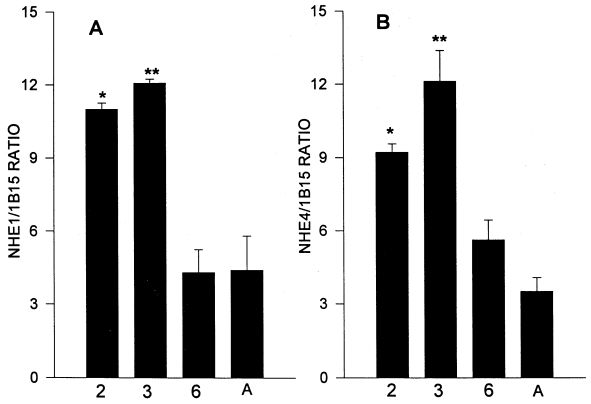

Fig. 3. Graphical representation of Northern blot data. Northern blot data for experiments which showed differential mRNA expression between age groups is presented graphically. Panel A depicts data from NHE-1 probes in rat kidney, and panel B depicts data from NHE-4 probes in rat kidney. The Y axis shows NHE/1B15 ratio in phosphorimage units. Two is two week rats, 3 is three week rats, 6 is six week rats, and A is adult rats. Panel A * and * $p < 0.01$ between 2 week and 6 week, 2 week and adult, 3 week and 6 week and between 3 week and adult; panel B * $p < 0.05$ between 2 week and adults, ** $p < 0.01$ between 3 week and 6 week, and between 3 week and adults.

week animals, 2 and 6 week animals, or 6 week animals and adults. Data from Northern blots with NHE-4 probes is presented graphically in Fig. 3. No NHE-4 message was detected in rat jejunum.

3.3. RT-PCR analysis of rat jejunum with NHE-2 and NHE-4 primers

Results of these experiments confirmed results of Northern blot analyses in that NHE-2 mRNA was detected, but no NHE-4 mRNA was detected with either primer pair (data not shown). Expected sized bands were present in all positive controls, and negative controls showed no DNA amplification.

3.4. Characterization of NHE-3 antiserum

NHE-3 antiserum was initially characterized by reacting it with protein from PS120 cells that had been transfected with NHE-2 or NHE-3 (Fig. 4). Results showed recognition of a single protein band at ≈ 85 kDa. This band was blockable by pretreatment of serum with antigenic protein (0.4 mg/ml for

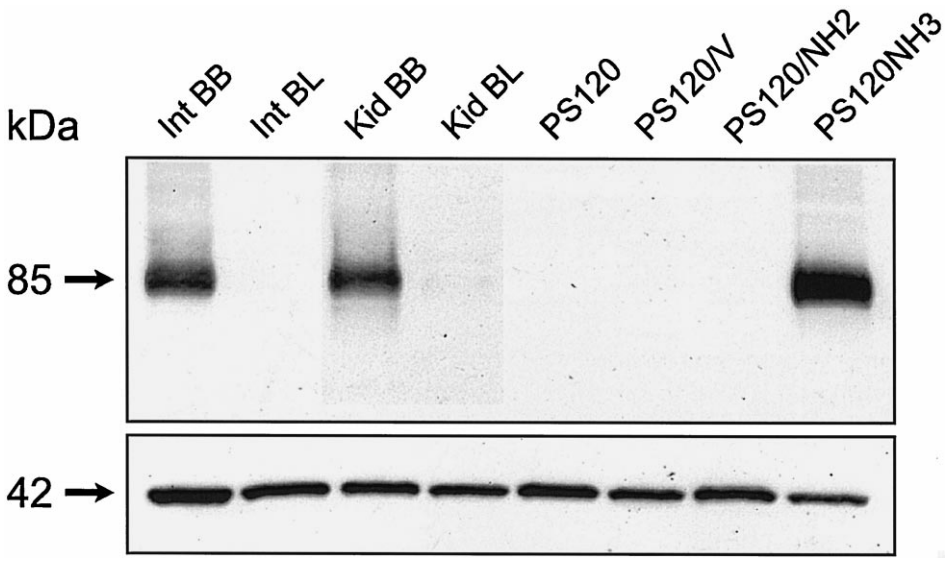

Fig. 4. Characterization of NHE-3 antiserum. Polyclonal antiserum was characterized by immunoblot analysis with BBM and BLM proteins (20 μg/lane) from rat kidney and jejunum and with PS120 cells transfected with NHE isoforms. The band at 85 kDa represents NHE-3 and the band shown below at 42 kDa is β-actin. Int BB is intestinal BB membrane proteins, Int BL is intestinal basolateral membrane proteins, Kid BB is renal BB membrane proteins, Kid B is renal basolateral membrane proteins, PS120 are untransfected cell crude membrane proteins, PS120/V are vector only transfected cells, PS120/NH2 are NHE-2 transfected cells, and PS120/NH3 are NHE-3 transfected cells.

16 h at 10°C) and the band was not seen with pre-immune serum (data not shown). The band was not present in untransfected PS120 cells, vector only transfected PS120 cells or NHE-2 transfected PS120 cells. The presence or absence of NHE mRNAs was confirmed by RT-PCR analysis (not shown). Additionally, NHE-3 antiserum was reacted with renal and intestinal BB and BL membrane proteins (Fig. 4). These results showed recognition of a single protein band at 85 kDa in both BB protein samples, but no protein bands were detected in BL membrane protein samples.

3.5. Assessment of BBM protein contamination in BLM preps

In order to assess the amount of contamination of BLMV preps with BBMVs, immunoblots were performed with NHE-3 antiserum and crude homogenate or BLM proteins from the 4 ages of rats (Fig. 5). Results showed that the crude homogenate proteins from renal and intestinal tissue at all age groups showed a strong NHE-3 band that was not present in any of the BLM protein samples. Even extended exposures of the blots detected no NHE-3 protein in

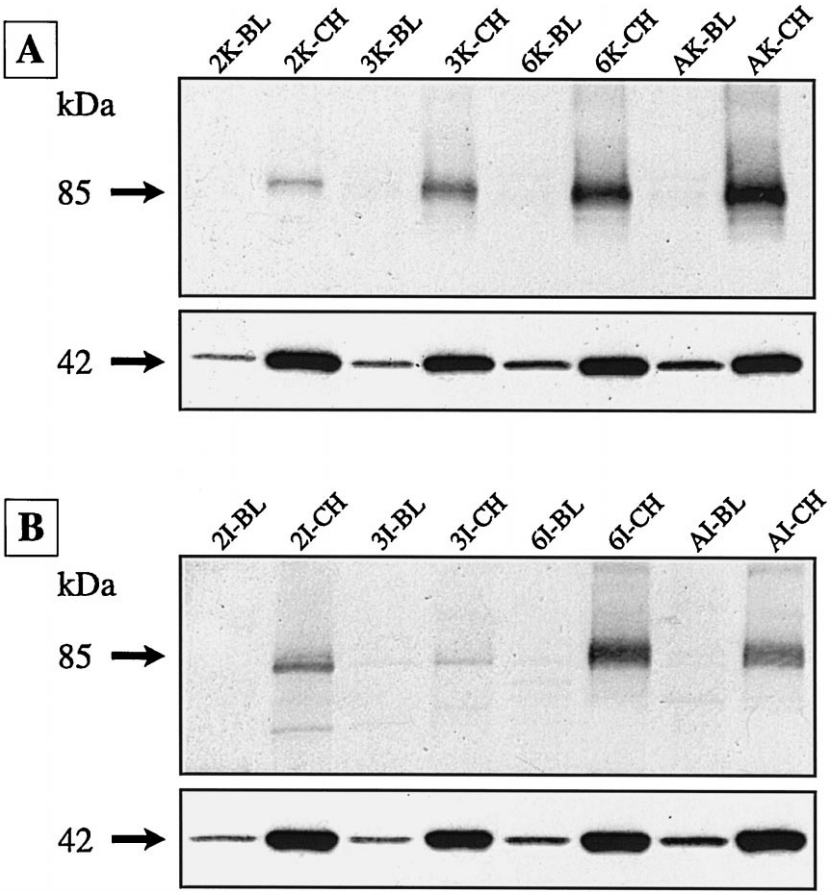

Fig. 5. Assessment of BBM protein contamination of BLM protein preps. Proteins were solublized from crude homogenates (CH) or basolateral (BL) membrane suspensions, fractionated by SDS/PAGE (20 μg/lane) and transferred to membranes. Membranes were reacted with NHE-3 polyclonal antiserum at 1:4000 dilution. The band at 85 kDa is NHE-3 and the band shown below at 42 kDa is β-actin which is shown to exemplify protein in each lane (β-actin is much more abundant in crude homogenate than in BLM fractions). Panel A shows antiserum reaction with renal samples and panel B shows antiserum reaction with jejunal samples. Panel A: 2 K is 2 week rat kidney; 3 K is 3 week rat kidney; 6 K is 6 week rat kidney; and AK is adult rat kidney. Panel B: 2 I is 2 week rat intestine; 3 I is 3 week rat intestine; 6 I is 6 week rat intestine; and AI is adult rat intestine.

the BLM protein preps (not shown). Additionally, immunoblots with monoclonal Na^+/K^+-ATPase antiserum showed a large enrichment in signal intensity in BLM proteins as compared to crude homogenate proteins and no signal in BBM proteins (data not shown). These observations confirm the results of Na^+/K^+-ATPase activity studies that showed an enrichment of 8–10-fold in BLM preps as compared to crude homogenates.

4. Discussion

The purpose of this investigation was to determine if previously observed ontogenic changes in BLMV NHE activity were due to differential expression of one of the BLM-specific NHE isoforms. Rats were studied at four different ages: 2 week at 14 days of age, 3 weeks at 21 days of age, 6 weeks at 42–45 days, and adults at 4–6 months of age. pH-dependent uptake of sodium by intestinal BLMV was assayed to confirm and expand upon earlier observations, and similar novel studies were performed in renal BLMV. Control immunoblot experiments showed that there was no contamination of BLM protein preps with the BBM specific protein, NHE-3. Slot blots were previously performed to determine the potential of probes generated from one NHE cDNA isoform to cross-hybridize with other NHE isoforms under our Northern blotting conditions [19]. Subsequently, Northern blots were performed with renal and jejunal poly $(A)^+$ RNA from the different age groups, utilizing specific probes for NHE-1 and NHE-4.

Initial rate uptake studies in the jejunum showed highest pH-dependent uptake of ^{22}Na in adult rats with a 2.8-fold decreases in 6 week rats, a 4.5-fold decrease in 3 week rats, and an 8.2-fold decrease in 2 week rats (all values as compared to adult values). All groups were significantly different from adult values, but no differences were noted between 6, 3, and 2 week animals. Earlier observations by our group showed highest initial rate uptake values in rat ileum in 6 week animals with $\approx$2.2-fold decrease seen in 3 and 2 week animals (6 week $\sim$ 1.95 nmol/mg protein/60 s, 3 week $\sim$0.9 and 2 week $\sim$0.85) [18]. These previous investigations determined that 30 sec uptake represented initial rate in all groups and that there were no differences between age groups in the following experimental parameters: enrichment of membrane preparations for basolateral membrane markers (Na^+/K^+-ATPase), impoverishment of membrane preps for mitochondrial membrane markers (cytochrome C oxidase) and endoplasmic reticulum membrane markers (NADPH cytochrome C reductase), level of Na^+ binding to vesicles as opposed to uptake into intravesicular space, affinity of the exchanger for Na^+ (K_m), and rate of pH dissipation and generation of negative membrane potential. These observations suggested that the phenomenon observed utilizing this method is based solely on the activity of the basolateral membrane NHEs at all age groups studied. These previous uptake values represent significant differences between 6 week values and those of 3 and 2 week animals. No differences were noted between 2 and 3 week animals. In contrast, the current investigation found no statistical differences between the values from these three age groups, although the numbers appeared to show differences. These discrepancies are most likely due to tissue specificity as the previous studies used ileal BLMV and the current investigation used jejunal BLMV.

Additional experiments were performed to address the issue of contamination of BLMV preps with BBMV. NHE-3 antiserum was characterized previously [19], but certain aspects of the characterization were repeated in the current investigation to exemplify the specificity of the serum for NHE-3. The serum was shown to react with a protein in NHE-3 transfected PS120 cells and not in untransfected or NHE-2 transfected cells. It also reacted with a protein in BBM protein samples but not in BLM protein samples. The band was blockable with antigenic protein and it was not present in pre-immune serum. Additionally, the serum reacted with an apical protein in jejunal tissue sections by immunohistochemistry, while BLMs and other cells within the tissue were not stained with antiserum [19]. These facts make it seem highly probable that the serum is recognizing the correct protein.

The NHE-3 serum was subsequently utilized to address the issue of contamination of BLMV preps with BBMVs. NHE-3 has been definitively localized to the BBM by our lab and several other groups. Immunoblots with crude homogenate proteins and BLM proteins showed that NHE-3 was present at

high levels in crude homogenate proteins from all groups in both tissues, but was totally absent from BLM proteins. Additional experiments were performed with Na^+/K^+-ATPase antiserum. This serum showed a weak band in crude homogenates, a much stronger band in BLM proteins, and no band was detected in BBM proteins. These observations indicate that the BLMV preps were not contaminated with an appreciable amount of BBMVs, which suggests that the NHE activity observed in the current investigation was due solely to BLM isoforms.

Slot blot analyses were previously performed to test the potential for cross-hybridization of probes specific for one NHE isoform to another NHE isoform [19]. Results showed no cross-reactivity of NHE-1 or NHE-3 with other isoforms, but cross-reactivity was apparent between NHE-2 and NHE-4. These results were not surprising due to the fact that areas exist in NHE-2 and NHE-4 that show high nucleotide sequence similarity (one region, as depicted in Fig. 6, shows a 200 bp region with $>95\%$ nucleotide sequence similarity). Specific probes were developed for NHE-4 that showed no sequence similarity, and these probes were shown to not cross-react with NHE-2 on slot blots. This DNA fragment was used to generate probes for the Northern blot analyses.

Northern blot analyses utilizing poly $(A)^+$ RNA isolated from jejunal mucosa of groups of rats at the desired ages, showed hybridization with NHE-1 probes only. Specific NHE-4 probes did not recognize any messages in rat jejunum. However, other NHE-4 probes, which contained regions of high sequence similarity between NHE-2 and NHE-4, showed hybridization with rat jejunum at all ages. We, therefore, conclude that NHE-4 message is not expressed in the rat jejunum, and that previous observations are most likely related to cross-hybridization of NHE-4 probes with NHE-2 mRNA in this tissue [3]. NHE-1 probes showed no differences in hybridization intensity in jejunum between the 4 age group rats. It, therefore, seems likely that changes seen in jejunal BLMV uptake between adult rats and younger animals is not related to differential expression of NHE-1. Furthermore, these changes cannot be related to expression of NHE-4, since NHE-4 message was not detected in rat jejunum by Northern blot analysis or by RT-PCR. It then becomes possible that other unidentified isoforms of NHEs or functionally related proteins may be responsible for these ontogenic changes in BLMV sodium uptake. Alternatively, mRNA stability or post-translational modification of NHE-1 may play a role in these ontogenic changes noted in the activity of BLMV NHE, by affecting functional levels of NHE-1 protein.

Initial rate uptake in the kidney was similar in 2, 3 and 6 week animals, and was reduced by ≈ 2.4-fold in adults. Northern blot analyses of rat kidney at the 4 age groups with NHE-1 and NHE-4 probes showed similar results. NHE-1 probes in kidney showed similar message levels in 2 and 3 week animals and significantly lower message levels in 6 week and adult animals. NHE-4 probes showed highest message levels in 2 and 3 week animals, and lower

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NHE2    - CACAGCCTGTGCCATGACGATGAACAAGTATGTGGAAGAGAACGTGTCCC -1258
          |||||| |||||  |||| ||||| ||||| ||||||||||||||||||
NHE4    - CACAGCTTGTGCAGTGACAATGAAAAAGTACGTGGAAGAGAACGTGTCCC -1499

NHE2    - AGAAGTCCTACACGACCATCAAGTACTTCATGAAGATGCTGAGCAGCGTG -1308
          ||| ||| ||||||||||||||||||||||||||||||||||||||||||
NHE4    - AGACGTCGTACACGACCATCAAGTACTTCATGAAGATGCTGAGCAGCGTG -1549

NHE2    - AGTGAGACCCTCATCTTCATCTTCATGGGCGTGTCCACCGTTGGGAAGAA -1358
          || ||||||||||||||||||||||||||||||||||||||||||||||||
NHE4    - AGCGAGACCCTCATCTTCATCTTCATGGGCGTGTCCACCGTTGGGAAGAA -1599

NHE2    - CCATGAGTGGAACTGGGCTTTCGTCTGCTTCACCCTGGCCTTCTGCCTGA -1408
          |||||||||||| |||||||||||||||||||||||||||||||||| ||
NHE4    - CCATGAGTGGATCTGGGCTTTCGTCTGCTTCACCCTGGCCTTCTGCCAGA -1649
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Fig. 6. Nucleotide sequence alignment of NHE-2 and NHE-4. NHE-2 and NHE-4 cDNAs contain regions of high nucleotide sequence similarity. The area of highest similarity is depicted here. NHE-2 (on top) and NHE-4 (on bottom) are indicated on the left. Nucleotide numbers are shown on the right. Lines indicate matches and blank spaces indicate that the nucleotides at that position do not match. Similarity between these regions is $>95\%$. Specific probes designed for NHE-2 and NHE-4 do not contain this region of the cDNAs.

message levels in 6 week animals and adults. Significant differences were between 2 week animals and adults, between 3 and 6 week animals, and between 3 week animals and adults. These results suggest that ontogenic changes seen in renal BLMV uptake may be in part due to differential expression of NHE-1 and NHE-4. The pattern of functional NHE activity in renal BLMV parallels the expression of the NHE isoforms 1 and 4 in that expression is similar in 2, 3, and 6 week animals and much lower in adult animals.

In conclusion, the results presented here show ontogenic changes in expression of functional NHE activity at the rat renal and jejunal BLM, and changes in message expression of two BLM NHE isoforms. The strongest correlations are between adult animals and the other age groups. Adults have highest functional exchanger expression in jejunum and lowest exchanger expression in kidney, as compared to the other age group rats. In jejunum, BLMV NHE activity shows ontogenic changes with highest expression levels in adult animals. However, expression of NHE-1, the only known BLM isoform expressed in rat jejunum BLM, does not show any ontogenic changes. In kidney, functional exchanger activity is lowest in adults, and NHE-1 and NHE-4 message expression is also lowest in adults. Overall, these results suggest that renal and jejunal NHE activity is regulated during rat ontogeny, and that regulation in the kidney is related to differential expression of NHE-1 and NHE-4 mRNA. Further experimentation will be necessary to determine the exact regulatory mechanisms that are responsible for these observations.

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