



Amiloride-sensitive epithelial sodium channel subunits are expressed in human and mussel immunocytes

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Abstract

In this study, we examined the expression of epithelial Na⁺ channel (ENaC) subunits in human peripheral blood lymphocytes, human lymph nodes and molluscan immunocytes using non-radioactive in situ hybridization. The results showed that T lymphocytes express the ENaC gamma subunit mRNA, and B lymphocytes the ENaC beta subunit mRNA. Yet, the alpha subunit mRNA was not detected in either cell type. In molluscan immunocytes, all three homologous ENaC subunit mRNAs are present, and these data were also confirmed by RT-PCR and sequencing of the PCR products. These findings show evolutionary conservation of the expression of ENaC subunits in immunocytes of invertebrates to vertebrates. The observed differential expression patterns of ENaC subunits suggest that ENaC function may be regulated differentially in different types of human lymphocytes. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Human T lymphocytes; Human lymph nodes; Molluscan immunocytes; ENaC; RT-PCR; In situ hybridization.

1. Introduction

The concentration of sodium ions (Na⁺) is tightly regulated in cells and extra-cellular fluids and blood. Changes in Na⁺ concentrations are accompanied by flow of water under osmotic pressure. Consequently, Na⁺ fluxes play a major role in the regulation of extracellular volume and blood pressure [1,2].

Sodium ions pass from the extracellular fluid into epithelial cells via Na⁺ channels located on the luminal plasma membrane. From the epithelial cells, Na⁺ ions are extruded into the interstitial fluid and capillaries by the Na/K ATPase that resides in the basolateral membrane [1–3]. These two transport mechanisms maintain a low concentration of Na⁺ in the cell while the extracellular levels remain high.

The importance of the epithelial Na⁺ channels (ENaC) in ion transport has been demonstrated by findings that hereditary mutations leading to hyper-activity of the ENaC result in Liddle's syndrome of hypertension [4], while mutations that result in loss of function cause pseudohypoaldosteronism with severe salt loss [5–9].

ENaC in human epithelial cells is composed of a multimeric complex of three subunits named alpha, beta and gamma ENaC subunits [3,4,6]. The three subunits have similar structures and are evolutionarily related, as they share similar gene structures and show 26–32% amino acid sequence homology [10]. The subunits include a large extracellular loop located between two hydrophobic segments that anchor the proteins to the membrane [3,6]. These proteins are members of a large superfamily of ion channels with two hydrophobic segments [11].

In contrast to mammalian epithelial sodium transport, there is no information on the molecular biology of sodium channels expressed in invertebrates. The ENaC superfamily also includes the degenerin family of genes expressed in the nervous system

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of the nematode *Caenorhabditis elegans* [12,13], and a peptide-gated sodium channel identified in neurons from the snail *Helix aspersa* [14].

The structural organization and the stoichiometry of the subunits forming functional sodium channels have still not been definitively established. In reconstituted systems, each subunit can form homomeric complexes [15], but the maximum channel activity has been observed when all three subunits of ENaC are co-expressed [16,17]. Recent studies suggest that the channel may be composed of nine subunits [18].

The stoichiometry of the subunits forming sodium channels may vary in different cell types and show ontogenesis-dependent differential expression [19–23]. The expression of the subunits is also regulated by hormones, especially aldosterone, as well as other factors that modulate electrolyte homeostasis [1,24,25]. A major recent hypothesis is that aldosterone stimulation of ENaC activity emanates from changes in the intracellular distribution, activation or insertion of existing ENaC subunits into the apical membrane [26,27].

This study was undertaken to examine the expression of ENaC subunits in human lymphocytes and molluscan immunocytes using *in situ* hybridization. We selected a mussel species as a sea-living invertebrate organism that must have well developed systems for regulating sodium flux. Our results revealed that mussel immunocytes express distinct mRNA sequences homologous to all three human ENaC subunit mRNAs. In contrast, in human peripheral blood mononuclear cells and lymph nodes ENaC subunits were found expressed differentially in a cell specific pattern.

2. Materials and methods

2.1. Samples

Peripheral blood mononuclear cells were obtained from healthy human donors (after informed consent). Whole blood was diluted (1:1) in Hanks solution (GIBCO, Life Technology, Inc., Gaithersburg, MD, USA) without Ca^{2+} and Mg^{2+} , and mononuclear cells were separated using Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) gradients. The cells were washed twice in Hanks solution, resuspended in RPMI 1640 medium (GIBCO), counted and diluted to a final concentration of 5×10^6 cells/ml. CD3 T lymphocytes were then isolated using magnetic beads conjugated with anti-CD3 monoclonal antibody (mAb) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions (Fig. 1). Purity of cell population was $>95\%$, as revealed by flow cytometry. Human lymph nodes were collected with permission of the Human Investigation Committee. The material was rapidly frozen in liquid nitrogen and stored at -80°C until the experiments. The analysis of lymph nodes was performed in the medullar area, which is known to be particularly rich in B cells. Molluscan immunocytes were obtained from *Mytilus galloprovincialis* collected from rocks in the Adriatic Sea around Cattolica (Rimini, RN, Italy) and maintained in the laboratory. The hemolymph, taken from the posterior adductor muscle using a 2-ml syringe, was cytocentrifuged on a slide at 800 rpm for 2 min (Cytospin 2 cytocentrifuge, Shandon, UK), and the immunocytes were then air-dried.

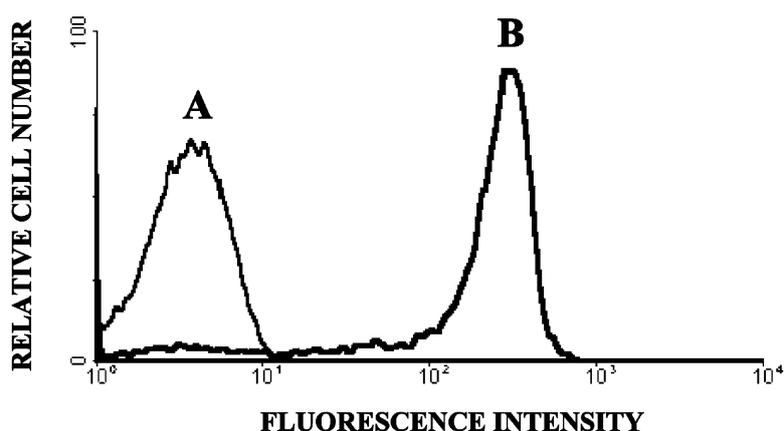


Fig. 1. . Expression of CD3 in lymphocytes isolated with magnetic beads conjugated with anti-CD3 mAb (B). One experiment representative of four is shown. Note that $>95\%$ cells were CD3+ T lymphocytes. Negative control (A).

2.2. ENaC probes

ENaC probes were generated by RT-PCR. RNA was extracted from cultured human blood lymphocytes using a single-step guanidinium thiocyanate acid/phenol/chloroform method with the Tri-reagent LS kit (Molecular Research Center, Inc., Cincinnati, OH, USA). Oligo(dT)15 primed cDNAs were synthesized from 1 to 5 µg RNA in a final volume of 20 µl using the First Strand cDNA Synthesis kit (Promega, Madison, WI, USA). An aliquot of first strand cDNA was then used in a subsequent PCR reaction with Taq polymerase and 0.5 µM of each primer. The primers and PCR conditions are listed in Table 1. After an initial denaturation step at 94 °C for 4 min, PCR was conducted for 35–40 cycles with denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 45 s and final extension at 72 °C for 7 min. The RT-PCR products were visualized by ethidium bromide staining on 12.5% polyacrylamide gels, purified using PCR-product purification kit (Boehringer Mannheim, Germany), and used as ENaC probes for in situ hybridization.

2.3. In situ hybridization

2.3.1. Dig-labeled probe preparation

The presence of mRNA ENaC subunits in human T lymphocytes, human lymph nodes and molluscan immunocytes was assessed using a non-radioactive kit (Cat. No. 1093 657, Boehringer Mannheim). The probes were labeled with digoxigenin (Dig) conjugated to deoxyuridine triphosphate by random primer DNA-labeling following Feinberg and Vogelstein [28]. The reaction was stopped by adding 0.2 M EDTA, pH 8.

Table 1
PCR conditions for generating the DNA fragments that were used for in situ hybridization

Subunit	Forward primer	Reverse primer	Size (bp)	MgCl ₂ (mM)	Annealing (°C)
Alpha	ggtagcctccaccctggcatc	gccttggtgtgagaaacctctc	229	1.5	60
Beta	caccaactttggcttcagcc	gtccagcggctgcagacgcag	133	6.0	60
Gamma	gcagaaagccaaggagtgtg	gatctgttctcaacctgc	275	6.0	60

The DNA sequence fragments of molluscan ENaC subunits have been sent to GenBank.

2.3.2. Hybridization reaction

Human T lymphocytes, molluscan immunocytes and cryostat lymph node sections were used both unfixed and fixed (4% p-formaldehyde in Sorensen buffer, pH 7.2) for 10 min at 4 °C. Samples were then treated with phosphate buffered saline (PBS) containing glycine (0.7%) for 5 min, permeabilized with 0.3% Triton X-100 in PBS for 15 min at room temperature (RT), washed with PBS, and with 2×SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7) for 10 min, and then incubated for 1 h at RT with the pre-hybridization mixture (4×SSC, 40% formamide, 1×Denhardt's solution). Hybridization was performed overnight at 37 and 45 °C by adding the denatured Dig-labeled cDNA probe to the pre-hybridization mixture. Probe concentration in the hybridization buffer was 40 ng/µl.

2.3.3. Detection of hybridization reaction

Following hybridization, the samples were rinsed once in 2×SSC for 1 h at RT, once in 1×SSC for 30 min at RT, once in 0.5×SSC for 30 min at 45 °C, and once in 0.5×SSC for 30 min at RT. Subsequently, the sections were treated with blocking reagent solution [2% normal sheep serum+0.3% Triton X-100 in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5)] for 30 min at RT, incubated for 2 h at RT with sheep anti-Dig Fab fragments conjugated to alkaline phosphatase diluted 1:500 in buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100, and washed twice for 5 min in buffer 1 and twice for 2 min in buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) at RT. The enzymatic activity was revealed by incubating the samples at RT in the following medium: 45 µl nitroblue tetrazolium salt (75 mg/ml in dimethylformamide), 35 µl 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt (50 ng/ml in dimethylformamide), 10 ml buffer 3 and 2.4 mg levamisole (Sigma, St. Louis, MO, USA). The reaction was stopped in buffer 4 (10 mM Tris-HCl, 0.25 mM EDTA, pH 8). The hybridization reaction was repeated three times. The specificity of the reaction was confirmed by omitting the probes or pre-treating the samples with 1 mg/ml RNase A (Boehringer Mannheim) in Tris-HCl, pH 7.5 for 1 h at RT.

2.4. RT-PCR in molluscan immunocytes

Total RNA was extracted from immunocytes of *M. galloprovincialis* using the SV total RNA isolation kit (Promega). RNA quality and quantity was checked by gel electrophoresis as described by Sambrook et al. [29]. RT-PCR experiment was carried out using the RT-PCR Access System (Promega). The primers and PCR conditions are listed in Table 1. The lengths of the PCR fragments were estimated using Matrix software (Quantavision, Madison, USA). PCR fragments were sequenced using automatic ABI sequencer (Perkin-Elmer, USA) at Centro di sequenziamento ENEA 'La Casaccia' (Rome, Italy).

3. Results

The in situ hybridization procedure using digoxigenin-labeled cDNA probes revealed a strong positive hybridization signal in the cytoplasm of T lymphocytes (Fig. 2A) only with the ENaC gamma subunit probe, whereas lymphocytes from human lymph nodes showed strong signal only with ENaC beta subunit probe (Fig. 2B). In neither cell types was the presence of the alpha subunit sequence detected.

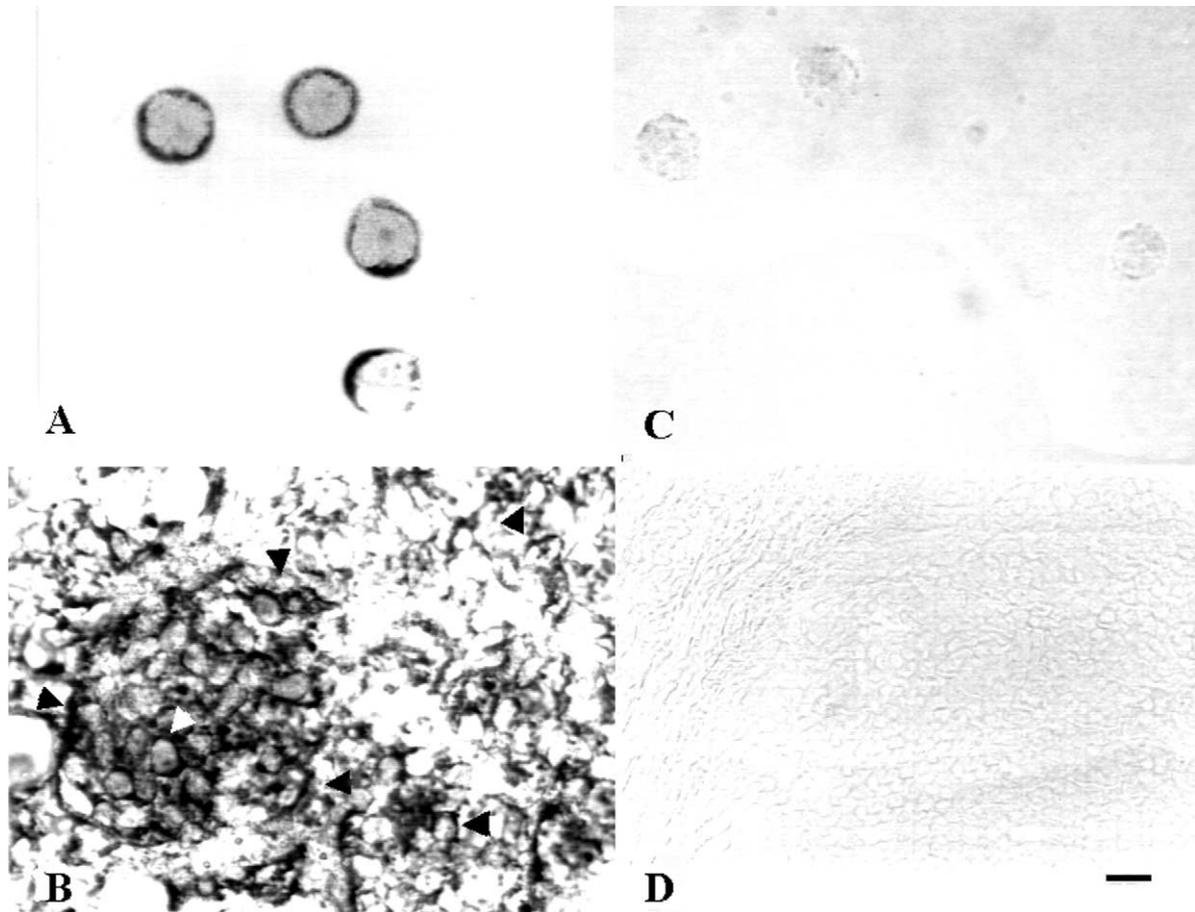


Fig. 2. . In situ hybridization using cDNA probes for alpha, beta and gamma ENaC subunits. Human T lymphocytes express only ENaC gamma subunit mRNA, while human lymph nodes express the ENaC beta subunit mRNA. (A) ENaC gamma subunit mRNA in human T lymphocytes; (B) ENaC beta subunit mRNA in human lymph node lymphocytes (arrowheads). The negative controls of in situ hybridization reactions were performed by omitting the probes or pre-treatment of the samples with 1 mg/ml RNase A: (C) human T lymphocytes and (D) human lymph node lymphocytes. Bar=10 μ m.

In the hemolymph of the mollusc *M. galloprovincialis*, only one cell type is present. This immunocyte reacts with antibodies recognizing epitopes present in molecules found on human lymphocytes, i.e. CD5, and on human NK cells, neutrophils and macrophages, i.e. CD11b and CD16 [30]. The in situ hybridization experiments showed the presence of mRNAs homologous to all three subunit sequences in immunocytes (Fig. 3A–C). No signal was seen in the negative controls of the in situ hybridization both in human lymphocytes (Fig. 2C and D) and molluscan immunocytes (Fig. 3D).

To confirm the identity of the in situ hybridization signals in the molluscan immunocytes, we carried out RT-PCR experiments using molluscan immunocyte mRNA and primers derived from the human subunit sequences (Table 1). These experiments yielded only a single strong band for each pair of primers (Fig. 4). The sizes of these fragments were estimated as 230, 130, and 270 bp, respectively, for the alpha, beta and gamma subunit primer sets. These sizes are similar to the sizes of the fragments obtained from the human mRNA (Table 1).

BLASTN [31] comparison of the molluscan PCR fragment sequences with the sequences in the GenBank database revealed that the sequences shared highest homology only with the respective ENaC subunits from humans and other species. The molluscan fragment generated using alpha ENaC primers showed 87% sequence identity within a 177 base fragment of the human ENaC alpha subunit gene. The fragment generated using beta ENaC primers showed 88% sequence identity within a 90 base fragment of the *Rana catesbeiana* ENaC beta subunit mRNA. The fragment generated using gamma ENaC primers showed 84% sequence identity within a 109 base fragment of the human ENaC gamma subunit gene.

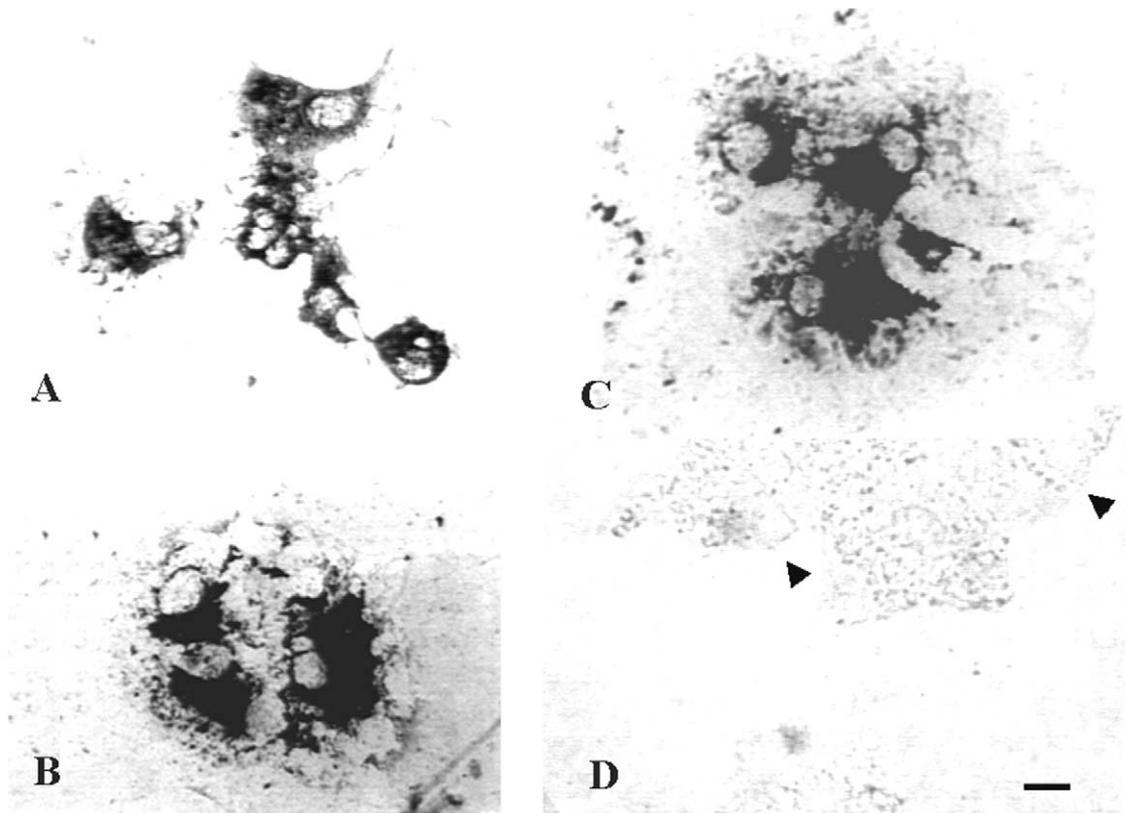


Fig. 3. . In situ hybridization using cDNA probes for alpha, beta and gamma ENaC subunits. All the subunits are present in molluscan immunocytes: (A)–(C) ENaC alpha, beta and gamma subunit mRNAs, respectively. The negative controls of in situ hybridization reactions were performed by omitting the probes or pre-treatment of the samples with 1 mg/ml RNase A: (D) molluscan immunocytes (arrowheads). Bar=10 μ m.

Surprisingly, in all cases the molluscan fragments showed highest homology with ENaC gene segments encoding amino terminal regions of the subunits, while the primers were designed to match a carboxy terminal segment of the subunits. This suggests that the human primers may be recognizing a homologous region in the molluscan genes. Determination of the complete sequences of the molluscan cDNAs may help to elucidate this difference.

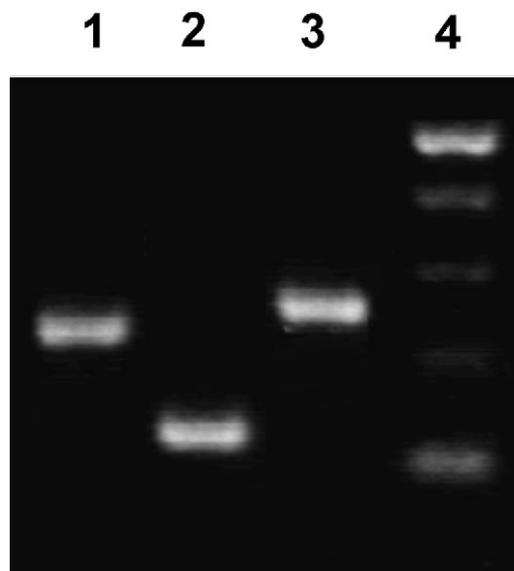


Fig. 4. . RT-PCR amplified fragments of ENaC subunit mRNAs in molluscan immunocytes. Lanes 1–3 show fragments obtained using alpha, beta and gamma subunit primer sets as shown in Table 1. The molecular weights of the fragments were estimated using the 100 bp ladder (lane 4).

4. Discussion

The findings of this study showed that mRNAs related to the ENaC subunits are expressed in both human lymphocytes and molluscan immunocytes. These findings represent the first direct evidence for the expression of homologous ENaC subunits in invertebrates. Liu et al. [32] have demonstrated that the degenerin family of proteins in *C. elegans* is homologous to subunits of the mammalian amiloride-sensitive epithelial sodium channels.

As a sea-living organism, mussels must have well developed systems to regulate sodium ion fluxes. Yet, until now there has been no molecular characterization of the proteins that fulfill this function. This study indicates that proteins homologous to mammalian ENaC subunits exist in mussels and probably other related organisms. The primary structure and functional roles of these proteins remain to be determined. Previous studies have reported that mammalian ENaC subunits show significant sequence similarities with those of the nematode *C. elegans*, *mec-4*, *mec-10*, *deg-1* and *unc-105* [12,13]. These genes of degenerin family are expressed in specific neurons and in muscle and are involved in sensory touch transduction and, when mutated, cause degeneration of specific sensory neurons. In addition, the amiloride-sensitive Na⁺ channel that is activated by FMRFamide and found in mollusc *H. aspersa* neurons is a new member of a channel superfamily including the ENaC [14].

The evolutionary conserved expression of ENaC subunits in lymphocytes and molluscan immunocytes suggest that amiloride-sensitive Na⁺ channels may also play a role in electrolyte homeostasis in these cells.

Recent studies by Bubien et al. [33] showed that amiloride completely inhibits sodium conductance in human lymphocytes indicating that amiloride-sensitive ENaC is the principal channel for sodium conductance in these cells. The activity of these channels is regulated by cAMP [33]. In several cases of the Liddle's syndrome, an abnormal Na⁺ channel activity has also been detected in peripheral blood lymphocytes [33]. Thus, the mutated ENaC subunit appears to be expressed in lymphocytes and the altered ENaC activity reflects the disease phenotype.

Our results in human peripheral lymphocytes and lymph nodes show that T lymphocytes express the ENaC gamma subunit mRNA and B-lymphocytes the ENaC beta subunit mRNA. In neither cell types was the alpha subunit detected. These findings do not exclude the expression of other subunits in small amounts. However, the strong in situ hybridization signals indicate that if other subunits were expressed they would represent only a small fraction of the total ENaC subunits. A previous study examined the expression of the beta subunit in different human B cell lines and human lymph nodes [34]. Our study has extended the findings to T lymphocytes, revealing a different pattern of expression. These results suggest that the function of ENaC may be modulated by different subunit compositions in different cell types. Prince and Welsh [35] have demonstrated that in two different mammalian cell lines, individual subunits expressed alone could move to the cell surface without assembling into a hetero-multimeric complex. Independent studies by different laboratories have shown that in mouse and rat kidney, alpha subunit expression is induced while the expression of beta and gamma subunits show little or no change [36–39]. These findings have led to the suggestion that ENaC activation may result from a change in subunit stoichiometry.

In conclusion, the data presented here indicate the presence of amiloride-sensitive Na⁺ channel subunit-like molecules at early stages of evolution. The differential expression patterns of the ENaC subunits in human lymphocytes may serve different functions.

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