Expression and Regulation of Normal and Polymorphic Epithelial Sodium Channel by Human Lymphocytes*

Received for publication, September 28, 2000, and in revised form, December 6, 2000
Published, JBC Papers in Press, December 11, 2000, DOI 10.1074/jbc.M008886200

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Gene expression, protein expression, and function of amiloride-sensitive sodium channels were examined in human lymphocytes from normal individuals and individuals with Liddle’s disease. Using reverse transcriptase polymerase chain reactions, expression of all three cloned epithelial sodium channel (ENaC) subunits was detected in lymphocytes. Polyclonal antibodies to bovine α-ENaC bound to the plasma membrane of normal and Liddle’s lymphocytes. A quantitative analysis of fluorescence-tagged ENaC antibodies indicated a 2.5-fold greater surface binding of the antibodies to Liddle’s lymphocytes compared with normal lymphocytes. The relative binding intensity increased significantly (25%; p < 0.001) for both normal and Liddle’s cells after treatment with 40 μM 8-CPT-cAMP. Amiloride-sensitive whole cell currents were recorded under basal and cAMP-treated conditions for both cell types. Liddle’s cells had a 4.5-fold larger inward sodium conductance compared with normal cells. A specific 25% increase in the inward sodium current was observed in normal cells in response to cAMP treatment. Outside-out patches from both cell types under both treatment conditions revealed no obvious differences in the single channel conductance. The P_{o} was 4.2 ± 3.9% for patches from non-Liddle’s cells, and 27.7 ± 5.4% in patches from Liddle’s lymphocytes. Biochemical purification of a protein complex, using the same antibodies used for the immunohistochemistry, yielded a functional sodium channel complex that was inhibited by amiloride when reconstituted into lipid vesicles and incorporated into planar lipid bilayers. These four independent methodologies yielded findings consistent with the hypotheses that human lymphocytes express functional, regulatable ENaC and that the mutation responsible for Liddle’s disease induces excessive channel expression.

Numerous cell types and tissues express amiloride-sensitive sodium channels. The most extensively studied are the toad urinary bladder (1, 2), the mammalian renal cortical collecting duct (3, 4), and the toad renal cell line (A-6) (5, 6). A prominent physiological function of these cell types is the vectorial transport of Na^+ and water. Epithelial cells of the lung also express amiloride-sensitive sodium channels. However, the specific function of these channels is less clearly defined than that of the salt-reabsorbing epithelia (7). Also, the biophysical and biochemical characteristics of lung epithelial cell amiloride-sensitive sodium channels are somewhat different from those of renal cells. These channels have a different affinity for amiloride, different unitary conductance, and different cationic selectivities (8–10). Amiloride-sensitive sodium channels have also been linked to taste (11). The current carried by these channels can only be partially inhibited by amiloride (12). The reasons for these differences are not clear. Possible explanations include multiple channel types derived from post-translational modifications of ENaC^1 or the expression of completely different gene products.

Human lymphocytes express a sodium conductance that is activated by cyclic AMP and inhibited by low concentrations of amiloride (IC_{50} = ~ 75 nm) (13). A sodium conductance with the same properties has been observed in whole cell clamped, principal cells isolated from the renal cortical collecting duct of the rat (14). The pharmacological and regulatory properties of the lymphocyte and renal principal cell channels suggest that they may be the same protein complex. Also, whole cell clamped lymphocytes isolated from individuals with Liddle’s disease have a constitutively activated inward sodium conductance (15). The addition of cyclic AMP to these whole cell clamped cells has no effect on the sodium conductance. In contrast, whole cell clamped lymphocytes from unaffected family members do not exhibit any constitutively active sodium current, even at strong hyperpolarizing voltage-clamp steps (i.e. -140 to -160 mV). The apparent constitutive activation of the Na^+ conductance could account for the hypertensive pathophysiology associated with Liddle’s disease.

Despite these biophysical similarities and the obvious abnormalities in sodium conductance regulation observed in whole cell clamped lymphocytes from individuals with Liddle’s disease, there has been some question as to the identity of the protein responsible for the amiloride-sensitive sodium conductance of lymphocytes. To resolve this question, we tested the hypothesis that lymphocytes express the ENaC by four independent methods (electrophysiological, immunocytochemical, biochemical, and molecular biological). The findings from each method indicated that ENaC was expressed by human lymphocytes. Also, because amiloride inhibited all the sodium conductance, the findings ruled out the possibility that other sodium channels, such as the voltage-gated sodium channel found in

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*This work was supported by National Institutes of Health Grants DK 37207 (to D. J. B.) and DK52789 (to J. K. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: ENaC, epithelial sodium channel; hENaC, human ENaC; PCR, polymerase chain reaction; RT, reverse transcriptase; bp, base pair(s); CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; DTT, dithiothreitol; MOPS, 4-morpholinopropanesulfonic acid.
nerve and muscle, were expressed by lymphocytes. This comprehensive investigation is the only set of data on ENaC of which we are aware that has utilized molecular biology, electrophysiology, protein chemistry, and immunofluorescence on a single cell type. The findings from all of these studies support the hypothesis that human lymphocyte ENaC has biophysical and regulatory characteristics that accurately reflect ENaC characteristics expressed by renal principal cells.

**EXPERIMENTAL PROCEDURES**

**Electrophysiological Methods**

Microelectrode pipette solutions contained 100 mM potassium-glucconate, 30 mM potassium chloride, 10 mM sodium chloride, 20 mM HEPES, 0.5 mM EGTA, 4 mM ATP, and <10 nM free calcium and were buffered to a pH of 7.2. All electrophysiological measurements were obtained with a bath solution of serum-free RPMI 1640 culture medium, buffered to a pH of 7.4, at room temperature (24 ± 2°C). Whole cell current records were obtained by formation of conventional whole cells. Briefly, after formation of high resistance seals between patch electrodes and lymphocyte plasma membranes (>3 Gohms), a sharp suction pulse was applied to rupture physically the plasma membrane under the seal, leaving the seal resistance unchanged. The increased cellular capacitance indicated the successful formation of the whole cell configuration. The capacitance was balanced using the appropriate circuits of the Axopatch 200 patch clamp amplifier (Axon Instrs.). The voltage clamp protocol was to hold the cells at −60 mV for 200 ms and clamp to membrane potentials ranging from −160 to +40 mV for 800 ms in sequences of 200 mV increments, returning to the holding potential for 200 ms between each clamp step.

Single amiloride-sensitive sodium channels were recorded in the outside-out patch configuration. This configuration was chosen because amiloride only inhibits the channels from the extracellular face of the channels. In the outside-out configuration, the outside of the channels were exposed to the bath solution. Thus, superfusing the preparations with 2 μM amiloride-supplemented RPMI was used as a straightforward method for establishing the identity of the channels. All experiments were performed on Epstein-Barr virus-transformed non-Liddle’s cells (Daudi) or Epstein-Barr virus-transformed B lymphocytes from affected relatives (normal) were Epstein-Barr virus-transformed and cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C and 5% CO2 (95% air) in 75-cm2 tissue culture flasks. Cells were transferred to another tube and mixed with 1/10 volume of isopropanol. The colorless aqueous phase contained the RNA. The aqueous phase was transferred to a 1.5 ml microfuge tube and centrifuged at 12,000 × g (maximum) for 5 min at 4°C. After removal of the ethanol, the pellet was air dried for 30 min and dissolved in 30 μl of diethylpyrocarbonate water, to ensure RNase-free conditions, for 15 min at 60°C.

cDNA Synthesis and Gene-specific Amplification

First-strand syntheses were performed using a modification of the MasterAmp High Fidelity RT-PCR Kit protocol (Epicenter Technologies, Madison, WI). Briefly, 25 μl of MasterAmp 2X RT-PCR premix plus 2 μl (40 units/μl) of MMLV-RT Plus 50 ng of total RNA (prepared as above), and 100 pmol of the gene-specific reverse primer were combined in a total reaction volume of 50 μl. The mixture was incubated at 37°C for 1.5 h.

For PCR amplification of specific ENaC subunits (α, β, and γ), we used 10 μl of the reversed transcribed product for each specific gene as template in a 25-μl reaction volume containing 100 pmol of each specific subunit forward and reverse primer for 50 cycles. Primers were designed based upon GenBank sequences (α-subunit, SCNN1A, accession number Z92981; β-subunit, SCNN1B, accession number U16623; and γ-subunit, SCNN1G, accession number L36592). The specific PCR primer sizes were: α-forward, 5′TGG TGC AGT CGC CAT AAT C-3′, with an expected product size with cDNA of 257 bp; β PCR primers: forward, 5′-TGG TTG AGC TGC ATG CAT A-3′, and reverse, 5′-CTG GAA CCA CTC CAA CCT CTG GAT GTC-3′; and γ PCR primers: forward, 5′-TCA AGA AGA ATC TTC CCG TGA C-3′, and reverse, 5′-GGT GGT AGT GGA CCT CTT GGA TGG AAA CTG-3′, with an expected product size with cDNA of 237 bp. The gene-specific reverse primers were used for cDNA synthesis. As a control for our gene expression assay, primers for the α-subunit were designed to span introns between exons 5 and 6 and between exons 6 and 7. Thus, the expected size for the α-subunit using genomic DNA was 1023 bp. PCR products derived from α-subunit cDNA using the same primer set were expected to be 257 bp. Primers for the β-subunit were within a single exon. Given the partial and equivocal sequence data for the γ-sequence in GenBank, primer design was guided by published intron/exon boundaries (16). Primers known to reside in introns in used were tested for the presence of genomic DNA.

**PCR Parameters**

For the α-subunit, PCR reaction was performed in a final reaction volume of 25 μl using 7.5 μl of double-distilled H2O, 2.5 μl of 10× PCR buffer (final concentration, 1× (Promega); final concentration, 1.5 mM MgCl2), 200 μM each dNTP (2.0 μl), 100 pmol each of the α-forward (100 pmol) and α-reverse (100 pmers), 5 units Taq DNA polymerase (Promega), and 10 μl of template cDNA. Cycle conditions were 94°C for 5 min for 1 cycle followed by 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 50 cycles. The PCR reaction mix for the β-subunit and γ-subunit were the same except for the appropriate gene-specific primers. The PCR amplification conditions for β-subunit and γ-subunit were also the same as for the α-subunit except the annealing temperature for β was 63°C. A second round of PCR amplification was performed using 2 μl of the 50-μl reaction product as template for a total of 30 cycles.

**Sequencing**

PCR products for all subunits were gel purified using the commercially available QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Purified products were directly sequenced using the gene-specific forward or reverse primer. Sequencing was performed using the Applied Biosystems Inc. model 377 sequencer in the University of Alabama at Birmingham Microbiology Sequencing Core.

**Biochemical Methods**

**Protein Purification**—Lymphocytes isolated from blood samples collected from patients with Liddle’s syndrome (Liddle’s) and from unaffected relatives (normal) were Epstein-Barr virus-transformed and grown in continuous culture in RPMI 1640 + 10% fetal bovine serum at 37°C and 5% CO2 (95% air) in 75-cm2 tissue culture flasks. Cells were collected by centrifugation at 500 × g for 5 min at 4°C. All subsequent procedures were carried out at 4°C. The lymphocytes were washed twice with phosphate-buffered saline and centrifuged. The cell pellet DNA. The supernatant was transferred to a fresh tube, and the RNA was precipitated by the addition of 450 μl of isopropanol, incubated at room temperature for 10 min, and centrifuged for 10 min at 4°C. The supernatant was removed, and the RNA precipitate was washed once with 75% ethanol (at a rate of 1 ml/750 μl of Tri-Reagent) followed by resuspension in 12,000 × g (maximum) for 5 min at 4°C. After removal of the ethanol, the pellet was air dried for 30 min and solubilized in 30 μl of diethylpyrocarbonate water, to ensure RNase-free conditions, for 15 min at 60°C.

For the γ-subunit, PCR reaction was performed in a final reaction volume of 25 μl using 10 μl of the reversed transcribed product for each specific gene as template in a 25-μl reaction volume containing 100 pmol of each specific subunit forward and reverse primer for 50 cycles. Primers were designed based upon GenBank sequences (α-subunit, SCNN1A, accession number Z92981; β-subunit, SCNN1B, accession number U16623; and γ-subunit, SCNN1G, accession number L36592). The specific PCR primer sizes were: α-forward, 5′TGG TGC AGT CGC CAT AAT C-3′, with an expected product size with cDNA of 257 bp; β PCR primers: forward, 5′-TGG TTG AGC TGC ATG CAT A-3′, and reverse, 5′-CTG GAA CCA CTC CAA CCT CTG GAT GTC-3′; and γ PCR primers: forward, 5′-TCA AGA AGA ATC TTC CCG TGA C-3′, and reverse, 5′-GGT GGT AGT GGA CCT CTT GGA TGG AAA CTG-3′, with an expected product size with cDNA of 237 bp. The gene-specific reverse primers were used for cDNA synthesis. As a control for our gene expression assay, primers for the α-subunit were designed to span introns between exons 5 and 6 and between exons 6 and 7. Thus, the expected size for the α-subunit using genomic DNA was 1023 bp. PCR products derived from α-subunit cDNA using the same primer set were expected to be 257 bp. Primers for the β-subunit were within a single exon. Given the partial and equivocal sequence data for the γ-sequence in GenBank, primer design was guided by published intron/exon boundaries (16). Primers known to reside in introns in used were tested for the presence of genomic DNA.
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was resuspended in Dounce buffer (10 mM Tris-Cl, pH 7.6, 0.5 mM MgCl₂, supplemented with protease inhibitors: 2 μg/ml DNase, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride) and incubated 10 min on ice. This suspension was homogenized for 30 strokes in a Dounce homogenizer. Tonically respiring buffer (10 mM Tris-Cl, pH 7.6, 0.5 mM MgCl₂, and 600 mM NaCl) was added to a final NaCl concentration of 150 mM. The homogenate was then centrifuged at 500 × g for 5 min, and the pellet was discarded. 0.5 mM EDTA, pH 8.0, was added to the supernatant to achieve a final concentration of 5 mM and then centrifuged at 100,000 × g for 45 min. The final pellet was solubilized overnight in a solution of 10 mM NaH₂PO₄, pH 7.4, 10 mM CHAPS, 10% glycerol, and protease inhibitors (no DNase).

For affinity purification, 5 mg of a polyclonal antibody raised against a bovine kidney papillary Na⁺ channel protein complex was irreversibly bound to protein A immobilized on agarose gel (Pierce). 2 ml of gel were first packed in a plastic column and equilibrated with 50 mM sodium borate, pH 8.2. The gel was resuspended in a solution of the IgG and incubated at room temperature for 30 min. Free antibody was washed from the gel with borate buffer. Bound IgG was cross-linked to the protein A by suspending the gel in a 6.6 mg/ml solution of dimethylpimelimidate (Pierce) for 1 h at room temperature. Unreacted imidate groups were blocked by incubating the column in 0.1 M ethanolamine, pH 8.2, for 10 min at room temperature. Lastly, the column was washed with 0.1 M glycine, pH 2.8, and then borate buffer. Before affinity purification, the column was equilibrated with 10 mM sodium phosphate, pH 7.5.

Solubilized lymphocyte membrane proteins were applied to either column, allowed to run into the gel bed, and incubated there for 1 h at room temperature. The column was drained and washed with 10 mM sodium phosphate until the absorbance (A₀₋₂₈₀) of the wash from the column was the same as that of phosphate buffer alone. Bound protein was then eluted with 0.1 M glycine-HCl, pH 2.8, in 500-μl fractions that were neutralized with 1 M Tris, pH 8.0. Absorbance measurements at A₀₋₂₈₀ were used to determine which fractions contained antigen.

Iodination—To visualize the products, immunopurified proteins were diluted to 100 μl with phosphate-buffered saline prior to iodination, using 125I. Iodinated proteins were separated from unbound label on Sephadex G-25-150 columns, mixed with an equal volume of 2× sample buffer containing 50 mM dithiothreitol (DTT), and electrophoresed through 8% polyacrylamide gels. Gels were dried and exposed to x-ray film.

Reconstitution—To study the single channel activity of the normal and Liddle's protein preparations, they were reconstituted into proteoliposomes and incorporated into planar lipid bilayers. The protein-containing fractions eluted from the antibody column were concentrated to 100 μl and then combined with a mixture of 500 μg of phosphatidylethanolamine, 300 μg of phosphatidylserine, and 200 μg of phosphatidylcholine. Extracti-Gel D, previously washed with KCl buffer (400 mM KCl, 5 mM Tris-Cl, pH 7.4, 0.5 mM MgCl₂) was added, and the total volume was brought to 600 μl with KCl buffer. This mixture was incubated first at room temperature for 45 min and then at 4 °C for 16 h with agitation. Extracti-Gel D was allowed to settle out of the resulting proteoliposome suspension by gravity. The protein was aliquoted and stored at −80 °C.

Planar Lipid Bilayers—Planar lipid bilayers were made from a phospholipid solution containing a 2:1 mixture of diphytanoyl-phosphatidylcholine/diphytanoyl-phosphatidylserine in n-octane (final lipid concentration, 25 mg/ml). Bilayers were bathed with 100 mM NaCl containing 10 mM MOPS-Tris buffer (pH 7.4). All solutions were filter-sterilized. Current measurements were performed using a high gain amplifier circuit. Applied voltage is referenced to the trans-chamber, which was connected to the current-to-voltage converter, and therefore was at virtual ground. Acquisition and analysis of single channel recordings were performed using pCLAMP software and hardware (Axon Instruments, CA). Data were stored digitally and were filtered at 300 Hz with an 8-pole Bessel filter prior to acquisition at 1 ms/point. All the analyses were performed for single active channels. The records shown here are representative of 19 separate experiments. Amiloride sensitivity of the immunopurified channels in the bilayer was determined by the addition of the drug to the trans-solution of the bilayer.

RESULTS

Molecular Biological Analysis of Human B-Lymphocyte RNA—Using total RNA prepared from lymphocytes we have synthesized cDNA using gene-specific primers for the α-, β-, and γ-subunits of the human amiloride-sensitive epithelial sodium channel. Fig. 1 shows the PCR products obtained using gene-specific cDNA prepared from B-lymphocytes as template. To test the efficacy of our cDNA preparation method and to confirm that our PCR products were indeed derived from cDNA and not genomic DNA, we designed primers flanking two introns in the α-subunit. When the same PCR primers were used for amplification of a segment of the α-subunit using genomic DNA and cDNA, two bands of different but expected sizes were obtained: 1023 bp for the genomic DNA and 257 bp for the α-subunit cDNA. Based upon the intron/exon composition and/or the lack of a clear and complete sequence for the β- and γ-subunits, a similar strategy for these units was not productive. Particularly problematic was designing primers that yield a product size amenable to direct sequencing. However, when one of the primers known to occur within an intron was used in an attempt to amplify these subunits, no amplification was obtained: 1023 bp for the genomic DNA and 257 bp for the α-subunit cDNA. Sequencing was used as a final confirmation of the subunit segments amplified from cDNA. Direct sequencing revealed that we indeed amplified the α- and β-subunits. Our cDNA sequencing results for the γ-subunit were problematic. This problem persisted even though the same primer sets were used to amplify genomic DNA and the product was successfully sequenced. The reverse primer of the same primer set was used for the cDNA synthesis and yielded the expected size band. Attempts at resolution have included efforts to sequence in both forward and reverse directions and amplification with the original primer set and then using nested primers to reduce the

FIG. 1. RT-PCR products from lymphocyte mRNA using primers specific for each of the cloned ENaC subunits. The predicted size for each product is: α, 257 bps; β, 277 bps. Direct sequencing of the products confirmed that the α and β proteins were ENaC.
likelihood of any nonspecificity. Finally, we have considered the occurrence of a polynucleotide tract causing the Taq DNA polymerase drop-off as well as the presence of secondary structure. The addition of Me2SO was not useful. Additionally, alternative primer sets have been designed; however, there remains a persistent lack of sequence data. This was a consistent negative observation, irrespective of whether the primer set is the one described or alternative ones tested. This finding would suggest that there is little or no expression of the γ-subunit in B-lymphocytes.

Normal Human Lymphocyte Amiloride-sensitive Sodium Conductance—When human lymphocytes are whole cell clamped using “normal” ionic gradients, they exhibit a variety of ionic conductances. However, when the cells are hyperpolarized there is very little, inward current (Fig. 2, top left panel). Addition of the membrane permeant active analog of cyclic AMP (8-CPT-cAMP; final concentration, 40 μM) to the bath solution significantly (p < 0.05, n = 6) increased the inward conductance at hyperpolarizing clamp potentials by 25% (Figs. 2, top right panel, and 3). As shown previously, the inward current that was activated by cAMP treatment can be completely inhibited by 2 μM amiloride (13–15).

After formation of the whole cell configuration, slow withdrawal of the patch electrode often results in resealing of the membrane fracture, with the outside of the resealed membrane facing the bath solution. The result of this procedure is an isolated membrane patch in the outside-out configuration. For the study of ENaC single channels, this patch orientation is useful because amiloride inhibits these channels from the outside only. Thus, in the outside-out configuration, amiloride can be used to identify unambiguously amiloride-sensitive sodium channels, such as ENaC. When the outside-out configuration was formed on normal (i.e. non-Liddle’s) lymphocytes, no inward single channel currents were observed when the electrical potential gradient was directed inward, in six of six consecutive patches. This finding was consistent with the basal whole cell conductance, which was very small, even at strong hyperpolarizing potentials. However, single channels were observed in five of six outside-out patches formed from cells that were previously treated with 40 μM 8-CPT-cAMP and had large

![Amplitude Histogram](image1.png)
![Level 1 Dwell Times](image2.png)

**FIG. 2.** Normal cultured lymphocytes were whole cell clamped, and then treated with 40 μM 8-CPT-cAMP. The treatment activated inward currents. After activation, outside-out patches were formed, and single channel currents were recorded. These were completely inhibited by amiloride (2 μM). The middle panels show a recording from the same outside-out patch before (left) and after (right) superfusion with amiloride. Amplitude histogram analysis showed that each patch contained a minimum of five channels. Open dwell time analysis showed that the channels had relatively fast open-to-closed transitions (τ < 10 ms).
inward whole cell currents prior to the formation of the outside-out configuration (Fig. 2, middle left panel). These channels were completely inhibited by 2 μM amiloride (Fig. 2, middle right panel). Analysis of the single channel records yielded a single channel conductance of 10 pS (0.6 pA) at a potential of 60 mV; Fig. 2, bottom left panel). Also, the time spent in the open configuration was brief (τ < 10 ms; Fig. 2, bottom right panel), with relatively long closures between multiple channel bursts of opening.

Amiloride-sensitive Sodium Conductance of Liddle’s Disease Lymphocytes—The same electrophysiological analysis was performed on lymphocytes from individuals with Liddle’s disease, with somewhat different results. All of the cells examined had a constitutively activated inward conductance at hyperpolarized clamp potentials (Fig. 3, top panel). Unlike non-Liddle’s cells, treatment with 8-CPT-cAMP did not significantly alter the whole cell currents. Another difference between Liddle’s lymphocytes and non-Liddle’s lymphocytes was observed after formation of outside-out patches. Single channel currents were observed in five of six outside-out patches made on Liddle’s lymphocytes, without pretreatment with 8-CPT-cAMP (Fig. 3, middle left panel). More than 90% of this single channel activity was inhibited by 2 μM amiloride (Fig. 3, middle right panel), as determined by amplitude histogram analysis (Fig. 3, bottom panel). The amplitude histogram also revealed that the single channel conductance (~10 pS; 0.65 pA at 60 mV) was not different from that measured for amiloride-inhibitable single channels in outside-out patches from non-Liddle’s cells.

The basal inward currents from Liddle’s cells and the cAMP-activated inward currents from non-Liddle’s cells typically had a “ragged” appearance. The corresponding single channel records typically showed bursts of rapid openings and closures from multiple channels within the patches. The whole cell current characteristics and single channel characteristics were compared by summing 30 sequential 800-ms segments of single channel activity at each clamp potential to produce a “virtual” whole cell current. The parameters underlying this analysis were that the patch contained eight channels, and that each cell contained ~716 ± 264 channels within the plasma membrane. The channel number was estimated from a conventional [14C]amiloride binding study (assuming 1:1 [14C]amiloride: ENaC binding) shown in Fig. 4. The same [14C]amiloride binding study was performed on Liddle’s lymphocytes. In these cells, a significantly higher (p = 0.04, n = 10) number of binding sites were found (2488 ± 751). This finding is consistent with the hypothesis that the Liddle’s mutation induces
excessive channel expression.

Using this information, the summed single channel records were multiplied by 3.3 (800 total/30 segments \times 8 channels/patch) to approximate the current that would be carried by 800 channels. It was found that the summed single channel currents and the actual whole cell currents were indistinguishable in both magnitude and morphology, supporting the hypothesis that the amiloride-inhibitable single channels were the current carriers for the amiloride-inhibitable inward whole cell current. Fig. 5 shows the results of this comparison.

**Immunohistochemical Analysis of Human Lymphocyte ENaC**—When non-Liddle’s disease human lymphocytes were treated with antibodies specific for bovine ENaC, they exhibited considerable specific immunofluorescence, indicating expression of epitopes that were recognized by the antibodies (Fig. 6, left panel). The staining was punctate and intracellular with some staining at the cell surface. In contrast, Liddle’s lymphocytes, treated identically, were brighter, and more fluorescence was concentrated at the plasma membrane. Concurrently, cells of both types were treated for 5 min with 40 \mu M 8-CPT-cAMP. The fluorescence intensity at the cell surface of more than 60 cells from each group was measured. It was found that untreated Liddle’s cells were 2.5 times more fluorescent than non-Liddle’s cells. Also, treatment with cAMP significantly (p < 0.05) increased the cell surface fluorescence intensity of both groups of cells. The quantitation of the relative fluorescence intensity of these cells is shown in Fig. 7. The most straightforward interpretation of these findings is that cells with the Liddle’s mutation express many more Na\(^{+}\) channels. This finding agrees reasonably well with the \([^{14}C]^{-}\)amiloride binding data, which showed a 3.47-fold difference between non-Liddle’s and Liddle’s lymphocytes, with Liddle’s lymphocytes expressing the higher number of binding sites. However, these findings do not rule out the possibility that more epitopes are exposed in Liddle’s cells because the exact number of epitopes is not known and because the channel stoichiometry is not known. This possibility is somewhat unlikely, however, because the immunofluorescence and \([^{14}C]^{-}\)amiloride binding results are in reasonable agreement with respect to the difference in channel expression between Liddle’s and non-Liddle’s lymphocytes. One other finding from the quantitation of the immunofluorescence was that cAMP treatment significantly increased the relative fluorescence intensity by 25% in each group. Again, there is no way from these experiments to distinguish between the possibility that more channels were inserted in the membrane or more epitopes of nascent channels were exposed by the treatment. There was no significant increase in the current in Liddle’s cells in response to cAMP treatment. However, the cell-to-cell variability was sufficiently large enough to preclude a statistically significant separation of only 25% in Liddle’s cells because of the constitutive activation of the channels and “ragged” morphology of the whole cell currents.

**Biochemical Analysis of Lymphocyte Sodium Channels**—Immunopurification from normal and Liddle’s cells yielded similar complexes of polypeptides, with molecular masses of 156, 79.5, and 68.5 kDa. The same banding profile was observed in the iodinated, reduced immunopurified complexes from normal...
and Liddle’s lymphocytes using the bovine kidney sodium channel antibody column (Fig. 8). These immunopurified polypeptide complexes were then assessed directly for their ability to function as sodium channels in planar lipid bilayer studies. The 79.5- and 68.5-kDa bands are consistent with α-, β-, or γ-ENaC (either glycosylated or unglycosylated), which typically run between 65 and 100 kDa. The larger band may correspond to a multimeric combination of these subunits. Others have purified bands of similar size from A6 epithelial cells and have speculated that this band was an xENaC multimer (19).

Unreduced immunopurified polypeptide complexes were incorporated into proteoliposomes and subsequently introduced into planar lipid bilayers to test the hypothesis that these complexes formed functional ion channels capable of conducting sodium. It was found that the complexes from normal lymphocytes and complexes immunopurified from Liddle’s lymphocytes were both able to conduct sodium in planar lipid bilayers. Consistent with the whole cell findings and with the pathophysiology of Liddle’s disease, the purified channels from Liddle’s cells spent a greater percentage of time in the open state compared with the immunoprurified channels from non-Liddle’s lymphocytes (Fig. 9). Also, amiloride inhibited the single channel currents recorded from these immunopurified polypeptide complexes (Fig. 9).

One obvious difference between the electrophysiological findings in intact lymphocytes and purified polypeptide complexes was in the efficacy of amiloride. Amiloride was approximately 1 order of magnitude less efficacious in inhibiting Na⁺ conductance in incorporated polypeptides than it was in inhibiting whole cell and single channel conductance in intact lymphocytes. Although the precise reason for this difference is unknown, it is reasonable to expect that the three-dimensional structure of the channel complex was somewhat altered by its removal from its native membrane and subsequent biochemical manipulation. Therefore, it is possible that the configuration of the amiloride-binding site was altered, resulting in a reduction in amiloride binding efficacy.

Another similarity between ENaC and the purified lymphocyte protein complex was the response to biochemical reduction using DTT. The addition of DTT to the trans-chamber caused protein complexes purified from both Liddle’s and non-Liddle’s lymphocytes to spend a much higher proportion of their conductive time in lower conductance states (Fig. 10). In bilayers, DTT reduced the single channel conductance from 35 to 13 ps, which is precisely the conductance observed in outside-out patches (Figs. 2 and 3). This same phenomena was observed when in vitro translated αENaC was subjected to DTT reduction in planar lipid bilayer preparations (17), and sodium channels was purified from bovine kidney using the same immunopurification procedure as was used on lymphocytes (18).
DISCUSSION

The hypothesis that the sodium conductance of human lymphocytes is mediated by a polypeptide complex containing the three cloned elements of the ENaC was tested by four independent methods. The findings from each method support the hypothesis that lymphocyte sodium conductance is accomplished via ENaC exclusively.

Reverse transcriptase polymerase chain reactions using ENaC-specific primers were amplified each ENaC subunit. The identities of αENaC and βENaC products were subsequently confirmed by direct sequence analysis of the cDNA. However, whereas the RT-PCR reactions using γENaC-specific primers yielded products of the correct size, subsequent sequence analysis failed to confirm the identity of these products. Thus, it remains unresolved as to whether or not γENaC plays a role in the function or regulation of the amiloride-sensitive sodium channels expressed by lymphocytes.

Our inability to demonstrate the presence of γ-hENaC by sequence analysis of lymphocyte RT-PCR products raises the possibility that lymphocytes express amiloride-sensitive sodium channels composed of only the α-hENaC and β-hENaC subunits. In oocytes it has been shown that sodium channels are formed by α-β, α-γ, and α-β-γ expression combinations (20–22). Thus, it is possible that lymphocytes express an α-β combination. However, as shown in Fig. 2, normal unstimulated lymphocytes have virtually no amiloride-sensitive sodium current. This is not consistent with reported constitutive activity of α-β channels (20). Also, the channels expressed by human lymphocytes appear to be highly regulated. They are activated by stimulation of α1-adrenergic receptors (23). They are activated by (14, 15, 24), and they are activated by aldosterone.

Further, lymphocyte amiloride-sensitive, whole cell sodium currents are electrophysiologically indistinguishable from the amiloride-sensitive, whole cell sodium currents found in rat

Z.-H. Zhou and J. K. Bubien, submitted for publication.
renal principal cells (14).

Because α-β, α-γ, and α-β-γ expression combinations all produce amiloride-sensitive sodium currents in oocytes, it is possible that any or all of these combinations are expressed by lymphocytes and principal cells. However, the most likely possibility is that principal cells express α-β-γ channels. This is because mutations in the β-subunit are known to produce Liddle’s disease (25), and a truncation mutation of the γ-subunit has been linked to familial low renin hypertension in one family (26). Oocyte studies indicate that maturation and assembly of subunits into channels is a “slow inefficient process” (21). Thus, there may be considerable variability of the mRNA levels, and that could be responsible for our inability to demonstrate γ-hENaC directly. Because of the similarity in current morphology, magnitude, response to agonists, and pharmacological profile of amiloride and its analogs (13) between the amiloride-sensitive whole cell sodium currents in lymphocytes and principal cells, it seems likely that both cell types express similar channels. Because the differences induced by the prototypical Liddle’s mutation produce an excess of channel expression in both oocytes and lymphocytes, the current evidence supports the hypothesis that both cell types respond similarly to mutation of β-hENaC. Thus, despite our inability to directly sequence γ-hENaC from lymphocyte mRNA, the other pieces of information currently available are consistent with the hypothesis that lymphocytes and principal cells express the same type of amiloride-sensitive sodium channels.

Electrophysiological examination of lymphocytes revealed a sodium conductance regulated by cAMP, and the activated current was completely inhibited by 2 μM amiloride. Obvious abnormalities were observed in this conductance when normal lymphocyte currents were compared with those obtained from lymphocytes obtained from individuals known to have a mutated ENaC β-subunit, (i.e. lymphocytes from individuals with Liddle’s disease). Liddle’s lymphocytes had a constitutively activated sodium conductance, whereas non-Liddle’s lymphocytes did not. This finding alone indicates that minimally, the β-ENaC subunit plays a role in lymphocyte sodium conductance, because the only difference between Liddle’s and non-Liddle’s lymphocytes is the mutation in the β-ENaC subunit. Two other electrophysiological findings further our understanding of lymphocyte sodium conductance. There was no difference in the single channel conductance. Thus, mutation of the β-ENaC subunit does not appear to affect the conductive pore of the channels. However, the kinetic activity of the channels expressed by normal cells appeared to be considerably less frequent than the channels expressed by Liddle’s cells. The basal whole cell currents in Liddle’s cells are significantly larger than the basal currents observed in normal non-Liddle’s lymphocytes. The difference could result from different numbers of channels (suggested by the greater plasma membrane immunofluorescence and greater number of [14C]amiloride binding sites) or different frequencies of opening and closing. Because single channel openings were rare events in membrane patches from non-Liddle’s cells, there is no basis for comparison of basal single channel kinetics between non-Liddle’s and Liddle’s lymphocytes. To resolve this problem additional techniques were applied to lymphocytes, protein biochemistry, and immunofluorescence.

Comparative immunofluorescence and [14C]amiloride binding analysis of normal and Liddle’s lymphocytes provided one explanation for the electrophysiological differences between normal and Liddle’s lymphocyte, whole cell sodium currents. That Liddle’s lymphocytes had 2.5 times more immunofluorescence and 3.5 times more [14C]amiloride binding suggests that these cells expressed more channels. Also, the immunofluorescence cent images indicate that the majority of the fluorescence increase was located at the plasma membrane. This finding could account for the larger whole cell currents and the increased frequency of encountering active channels in outside-out patches. However, a finding of 2.5 times more channels does not fully explain the 4.5-fold increase in basal whole cell current. The explanation for this discrepancy was elucidated by examining single channel kinetics of immunopurified polypeptide complexes incorporated into planar lipid bilayers.

Removing ion channels from their native plasma membranes and the normal cellular signaling pathways provides two advantages over studying channels by patch clamp. First individual channels can be examined, because channel incorporation into planar lipid bilayers is a rare event. Typically vesicles are “forced” into the bilayers by applying a voltage across the bilayer and eliminating the voltage gradient once a vesicle fusion is observed and then promptly washing the remaining vesicle from the chamber. This procedure often results in only a single polypeptide complex being incorporated into the bilayer. Under these conditions, the intrinsic kinetic properties can be examined directly. Fig. 10 shows directly that in planar lipid bilayers stripped of all regulatory elements and cellular connections, normal channels remain mostly closed interspersed with brief openings. In contrast, Liddle’s channels remain mostly open interspersed with brief closures. This difference in intrinsic channel kinetics combined with the increased number of channels observed with immunofluorescence can fully account for the large basal inward sodium currents typically observed in whole cell clamped Liddle’s lymphocytes.

These studies provide a comprehensive examination of the hypothesis that human lymphocytes express functional ENaC that is highly regulated. We know of no other cell type or tissue in which all of these investigative techniques have been successfully applied to the study of ENaC. This comprehensive investigation demonstrates directly the utility of lymphocytes for the investigation of ENaC. The studies were performed on human cells, thereby eliminating differences between species. Lymphocytes are available from individuals with suspected ENaC pathophysiology such as pseudohyperaldosteronism by obtaining a small (15 ml) blood sample. Further, lymphocytes are readily transformed, providing a continuing source of viable cells that express suspected ENaC polymorphisms. These studies also demonstrated differences in both electrophysiological characteristics and protein expression between normal lymphocytes and lymphocytes from individuals with Liddle’s disease. All of the findings were consistent with the pathophysiology of the hypertensive disorder. Thus, in this well characterized disorder lymphocytes appear to reflect accurately the cellular physiology of the principal cells of the renal collecting duct, which produce the in vivo hypertensive pathophysiology.

REFERENCES

1. Frings, S., Purves, R. D., and Macknight, A. D., (1988) J. Membr. Biol. 106, 157–172
2. Garty, H., Warncke, J., and Lindemann, B. (1987) J. Membr. Biol. 95, 91–103
3. Schafer, J. A., and Troutman, S. L. (1986) Am. J. Physiol. 250, F1063–F1072
4. Schlatter, E., and Schafer, J. A., (1987) Pflügers Arch. Eur. J. Physiol. 409, 81–92
5. Eaton, D. C., and Hamilton, K. L. (1988) Ion Channels, 1, 251–282
6. Marunaka, Y., and Eaton, D. C. (1991) Am. J. Physiol. 260, C1071–C1084
7. Matalon, S., and O’Brochta, H. (1999) Annu. Rev. Physiol. 61, 627–661
8. Smith, P. R., and Benos, D. J. (1991) Annu. Rev. Physiol. 53, 509–530
9. Palmer, L. G. (1992) Annu. Rev. Physiol. 54, 51–66
10. Rossier, B., Canessa, C. M., Schild, L., and Horisberger, J. D. (1994) Curr. Opin. Neph. Hypertens. 3, 487–496
11. Avenet, P., and Lindemann, B. (1988) J. Membr. Biol. 105, 245–255
12. Lindemann, B. (1999) Curr. Topics Membr. 47, 315–336
13. Buhien, J. K., and Warnock, D. G. (1993) Am. J. Physiol. 265, C1175–C1183
14. Buhien, J. K. (1995) Am. J. Physiol. 269, C791–C796
15. Buhien, J. K., Ismailov, I. I., Bershidy, B. K., Cornwell, T., Lifton, R. P., Fuller, C. M., Achard, J.-M., Benos, D. J., and Warnock, D. G. (1996) Am. J. Physiol. 270, C208–C213
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16. Thomas, C. P., Doggett, N. A., Fisher, R., and Stokes, J. B. (1996) J. Biol. Chem. 271, 26062–26066
17. Ismailov, I. I., Awayda, M. S., Berdiev, B. K., Bubien, J. K., Lucas, J. E., Fuller, C. M., and Benos, D. J. (1996) J. Biol. Chem. 271, 807–816
18. Ismailov, I. I., Berdiev, B. K., Bradford, A. L., Awayda, M. S., Fuller, C. M., and Benos, D. J. (1996) J. Membr. Biol. 149, 123–132
19. Zuckerman, J. B., Chen, X., Jacobs, J. D., Hu, B., Kleyman, T. R., and Smith, P. R. (1999) J. Biol. Chem. 274, 23286–23295
20. Fyfe, G. K., and Canessa, C. M. (1998) J. Gen. Physiol. 112, 423–432
21. Valentijn, J. A., Fyfe, G. K., and Canessa, C. M. (1998) J. Biol. Chem. 272, 30344–30351
22. Zhang, P., Fyfe, G. K., Grchtchenko, I. I., and Canessa, C. M. (1999) Biophys. J. 77, 3043–3051
23. Bubien, J. K., Cornwell, T., Bradford, A. L., Fuller, C. M., DuVall, M. D., and Benos, D. J. (1998) Am. J. Physiol. 276, C1405–C1410
24. Bubien, J. K., Jope, R. S., and Warnock, D. J. (1994) J. Biol. Chem. 269, 17780–17783
25. Shimkets, R. A., Warnock, D. G., Bositis, C. M., Nelson-Williams, C., Hansson, J. H., Schambelan, M., Gill, J. R., Ulick, S., Milora, R. V., Findling, J. W., Canessa, C. M., Rossier, B. C., and Lifton, R. P. (1994) Cell 78, 407–414
26. Hansson, J. H., Nelson-Williams, C., Suzuki, H., Schild, L., Shimkets, R., Lu, Y., Canessa, C. M., Iwasaki, T., Rossier, B. C., and Lifton, R. P. (1995) Nat. Genet. 11, 76–82