Rabbit retinal neurons and glia express a variety of ENaC/DEG subunits

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Some members of the epithelial Na+ channel/degenerin (ENaC/DEG) family of ion channels have been detected in mammalian brain. Therefore, we examined the RNA and protein expression of these ion channels in another part of the central nervous system, the retina. Because there are no physiological studies of amiloride-sensitive current in Müller glia, we next sought to demonstrate physiological evidence for an amiloride-sensitive current in Müller glia, which, on the basis of a previous study, are thought to express α-ENaC (Golestaneh N, de Kozak Y, Klein C, and Mirshahi M. Glia 33: 160–168, 2001). RT-PCR of retinal RNA revealed the presence of α-, β-, γ-, and δ-ENaC as well as acid-sensing ion channel (ASIC)1, ASIC2, ASIC3, and ASIC4. Immunohistochemical labeling with antibodies against α-ENaC and β-ENaC showed labeling in Müller cells and neurons, respectively. The presence of α-ENaC, β-ENaC, and ASIC1 was detected by Western blotting. Cultured Müller cells were whole cell patch clamped. These cells exhibited an inward Na+ current that was blocked by amiloride. These data demonstrate for the first time both the expression of a variety of ENaC and ASIC subunits in the rabbit retina as well as distinct cellular expression patterns of specific subunits in neurons and glia.

epithelial sodium channel; amiloride; retina; reverse transcriptase-polymerase chain reaction; patch clamp

MEMBERS of the epithelial Na+ channel/degenerin superfamily (ENaC/DEG) include the ion-gated, amiloride-blockable channels located in apical membranes of many salt-reabsorbing epithelia. The cloning of ENaCs has revealed the identity of α (6, 22)-, β- (7, 21)-, γ (7)-, and δ (38)-subunits. Other family members include the acid-sensing ion channels (ASICs) or brain Na+ channels (BNaCs) (2, 15, 31, 37, 39). Caenorhabditis elegans DEGs (10), the Phe-Met-Arg-Phe-NH2-gated channel (FaNaC) of Helix aspera (21), as well as Pickpocket (PPK) and Ripped Pocket (RPK) genes of Drosophila melanogaster (1).

Characteristic properties of the proteins in this superfamily include two transmembrane domains, a large extracellular loop with numerous glycosylation sites, and cytoplasmic amino and carboxy termini. Although the actual channel stoichiometry is debated, evidence exists in favor of heteromultimers (7, 13, 14). ENaC expression has been demonstrated in a variety of tissues including the retina. Matsuo (23) found expression of the α-subunit in the retinal pigment epithelium (RPE), ganglion cells, inner nuclear layer (INL), and outer nuclear layer (ONL) of rat retina by in situ hybridization. Mirshahi et al. (28) used a polyclonal antibody that recognizes a region of the α-subunit from rat colon and localized it in outer and inner segments of rods and cones, ganglion cells, inner plexiform layer (IPL), outer plexiform layer (OPL), INL, ONL, and RPE of rat and human retina. Mirshahi et al. (26) revealed α-ENaC expression in cultured bovine retinal pigment epithelium, and Golestaneh et al. (17) demonstrated expression of α-ENaC in cultured rat Müller glia.

Our aim was twofold. First, we wanted to expand on the previous work that localized the α-subunit by determining the pattern of expression in Müller cells. These cells have distinct patterns of expression of other ion channels and transporters. For example, inward-rectifying K+ channels facilitate the exchange of K+ between extracellular retinal space and the vitreous (29). One role of amiloride-sensitive channels is the regulation of Na+ homeostasis. Thus we hypothesized that the subcellular localization of ENaC in Müller cells might provide evidence to support the notion that they are involved in Na+ homeostasis in the retina. Because there are no physiological studies of amiloride-sensitive channels in the retina, we performed whole cell patch-clamp analysis with cultured Müller cells. We present here for the first time direct evidence for a functional ENaC channel in rabbit Müller glia by patch-clamp analysis.

Second, we sought to identify other subunits of this superfamily in the rabbit retina and to determine which cell types express specific subunits. Unique polyclonal antibodies against α-ENaC and β-ENaC were used to determine the distribution of these subunits in...
the rabbit retina by immunohistochemistry. In addition, double labeling with known retinal cell markers was used to identify specific retinal cell types expressing ENaCs. We also confirmed the presence of α-ENaC, β-ENaC, and ASIC1 proteins by Western blotting. Finally, subunit-specific primers were used in RT-PCR analysis of rabbit retinal RNA to test the presence of ENaC and ASIC mRNA.

EXPERIMENTAL PROCEDURES

All animal procedures followed institutional guidelines for the care and use of laboratory animals and the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings.”

Immunocchemicals. Polyclonal antibodies were raised in rabbit against the peptides KGKREEEGQLGPE of human α-ENaC (α-hENaC), GEKYCNRDF of human β-ENaC (β-hENaC), and DVKRHNPCELSLRGH of human ASIC1. Antisera were protein A purified and used at a dilution of 1:100. Mouse anti-tyrosine hydroxylase (TH) (Inostar, Stillwater, MN) at 1:800 was used as a marker for dopaminergic amacrine cells, goat anti-calretinin (Chemicon, Temecula, CA) at 1:800 for ganglion cells and AII amacrine cells, mouse anti-protein kinase C (PKC; Amersham, Little Chalfont, UK) at 1:100 for rod bipolar cells, goat anti-choline acetyltransferase (ChAT; Chemicon) at 1:200 for cholinergic cells, and mouse anti-vimentin (Dako, Carpinteria, CA) at 1:200 for Müller cells. Secondary antibodies (Jackson ImmunoResearch, West Grove, PA) included fluorophore-conjugated donkey anti-rabbit, donkey antimouse, and donkey anti-goat antibodies. Normal rabbit IgG was purchased from Jackson ImmunoResearch.

Characterization of α-hENaC, β-hENaC, and ASIC1 antibodies. Several experiments were performed to confirm the specificity of our anti-α-hENaC, anti-β-hENaC, and anti-ASIC1 antibodies. First, Madin-Darby canine kidney (MDCK) cells expressing rabbit α3γ-ENaC (rENaC) (R. G. Morris and J. A. Schafer, unpublished observations) were used as a positive control for Western blotting. Cell lysates from either MDCK cells expressing α3β-ENaC or nonexpressing parental MDCK cells were subjected to 8% SDS-PAGE and Western blotting. Blots were probed with 1:200 anti-α-hENaC or anti-β-hENaC. Second, we used the method of Jovov et al. (20) for in vitro transcription and translation and immunoprecipitation of α- and β-ENaC with our anti-ENaC antibodies. Both total in vitro translated protein and immunoprecipitates were subjected to SDS-PAGE and autoradiography. To test the specificity of anti-ASIC1, a lysate of human glioblastoma cells, U251-MG, was subjected to 7.5% SDS-PAGE and Western blotting. U251-MG cells express ASIC1 (D. J. Benos, unpublished observation) and thus were an appropriate positive control. Blots were probed with either anti-ASIC1 or normal rabbit IgG.

Immunohistochemistry of retinal sections. Eyes were enucleated from rabbits killed by an overdose of pentobarbital sodium (Socumb; Butler, Columbus, OH), and the retinas were quickly isolated. Retinas were fixed by several methods: periodate-lysine-1% paraformaldehyde (1% PLP) for 2 h at room temperature without vitreous, 2% paraformaldehyde at 4°C for 4 h both with and without vitreous, or 0.01% picric acid-2% paraformaldehyde at 4°C for 10 min with a 50-min postfix of 2% paraformaldehyde at 4°C without vitreous. Retinas were then cryoprotected and embedded in 50% optimum cutting temperature (OCT) solution (Tissue-Tek, Torrance, CA) and 50% Aquamount (Lerner Laboratories, Pittsburgh, PA) and cryosectioned in 10-μm slices.

Sections were stained with QuickStain (American Histology MasterTech Scientific, Lodi, CA) as an equivalent hematoxylin and eosin stain to visualize the retinal cellular layers. Conventional immunohistochemistry methods were used. Briefly, sections were blocked with a solution of 10% normal serum from the host of the secondary antibody, PBS, 0.3% Triton X-100, and 2% nonfat dry milk (NFDM) for 1 h at room temperature. All primary antibodies were diluted in PBS-Triton X-100-2% NFDM and 5% normal serum from the secondary antibody with an overnight incubation at 4°C. All secondary antibodies were diluted in PBS-Triton X-100-NFDM with a 1-h incubation at room temperature and protected from light. Slides were washed with PBS between steps and before coverslipping with Permafluar (Immuno, Pittsburgh, PA). Sections were viewed with fluorescence light microscopy or with a Leica TCS SP confocal laser scanning microscope. Double-labeled slides were scanned sequentially to avoid artificial bleed-through. Images were imported into Adobe Photoshop for figure preparation.

Müller cell isolation and culture. We followed the protocol of McGillem et al. (24) for the isolation and culture of rabbit Müller cells. Briefly, adult rabbits were killed and the eyes were enucleated and quickly cleaned. The cornea, iris, anterior segment, and vitreous were removed. The retina was hemisected and submerged in DMEM (Life Technologies, Rockville, MA) and EDTA (Sigma) for several minutes. The retinas were then gently teased away from the choroid and incubated in 5 ml of the above solution with 130 U of papain (Worthington, Lakewood, NJ) and 4.5 mg of cysteine (Sigma). After the enzymatic digestion, the retinas were placed in DMEM with 10% fetal bovine serum (FBS; Life Technologies) with 0.1 mg/ml DNase (Sigma) and triturated through a pipette. The dissociated cell preparation was placed in culture flasks or dishes coated with poly-l-ornithine (Sigma) and incubated overnight to allow the Müller cells to settle. The next day, the media were changed, and the cells were cultured and exchanged with plating medium (DMEM-10% FBS) and the unattached cells and debris were washed away. The result was a 90–95% pure population of Müller cells. Cells were maintained in a 5% CO2 37°C humidified incubator. Culture medium was changed every 2–3 days, and cells were split 1:2 with 0.25% trypsin-1 mM EDTA (Life Technologies) for immunohistochemistry or mechanically for patch clamping. One percent antibiotic-antimycotic solution (Life Technologies) was used after 1 wk as needed.

Immunohistochemistry of cultured Müller cells. This procedure was completed at room temperature on cells plated in 12-well plates on coverslips coated with poly-l-ornithine. All incubations except fixation were performed while shaking the plates. Washes with 0.1 M phosphate buffer (PB) occurred between steps. Culture medium was aspirated, and cells were washed three times with 0.1 M PB and fixed with 2% PLP for 1 h. Permeabilization and blocking were carried out for 30 min with 0.3% Triton X-100-PBS with 10% normal serum from the host of the secondary antibody. Cells were incubated in anti-α-hENaC or anti-vimentin, diluted 1:100 and 1:500, respectively, plus 5% normal serum for 1 h to overnight. Fluorophore-conjugated secondary antibodies with 5% normal serum were used for 1 h and protected from light. Coverslips were mounted on slides with Permafluar and viewed with fluorescence light microscopy or confocal microscopy.

Retinal protein extraction and Western blotting. Freshly enucleated rabbit eyes were submerged in ice-cold saline before dissection. The cornea, iris, anterior segment, and...
vitreous were removed, and the eyecups were placed in Cellgro-free medium (Mediatech, Herndon, VA) for several min- utes at 37°C to allow the retina to detach from the RPE. The retinas were teased away and homogenized on ice in radio-im- munoprecipitation (RIPA) buffer [1% Triton X-100, 0.1% SDS, 1% deoxycholate, 10 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 10 μg/ml leupeptin (Sigma), and 10 μg/ml pepstatin A (Sigma)]. The homogenate was spun, and the supernatant was precloled with nonimmunized rabbit IgG. Three parts precloled protein sample was incubated in one part SDS sample buffer, boiled at 100°C for 10 min, vortexed, and spun. The supernatant was cooled on ice before loading on the gels.

Standard electrophoresis and blotting protocols were fol- lowed. Briefly, protein was run on 5–15% gradient SDS-PAGE mini gels with 4% stacking gels for ~1 h at 150 V in a BioRad Miniubcell apparatus (Hercules, CA). Gels were transferred onto polyvinylidene fluoride (PVDF), treated with 2% NFDM-TBS-Tween, and probed with the appropri- ate ENaC antibody. An anti-rabbit secondary antibody was conjugated to horseradish peroxidase (Jackson Immuno-Research), and visualization was performed with chemilumi- nescent reagents (Amersham Pharmacia Biotech, Piscata- away, NJ). Controls included substitution of normal rabbit IgG for primary antibodies and/or omission of the primary antibody.

RNA extraction and RT-PCR. Freshly enucleated rabbit eyes were quickly submerged in RNAlater (Ambion, Austin, TX), and the retinas were isolated while minimizing the amount of RPE. Subsequent RNA isolation was performed with Stratagene’s Absolutely RNA RT-PCR Miniprep kit (La Jolla, CA), omitting liquid nitrogen freezing. Total RNA was extracted in 1 μl of enzyme solution, and each primer at 0.6 μM. Each reaction mixture consisted of 1.2 μg of RNA, 400 μM deoxyribonucleotide triphosphates, 5–10 U of RNAsin (Promega, Madison, WI), 2 μl of enzyme solution, and each primer at 0.6 μM. Reactions with water substituted for RNA were used as negative controls. The reactions were amplified with the GeneAmp PCR System 2400 (Perkin Elmer, Boston, MA). Reverse transcrip- tion was carried out at 50°C for 30 min followed by amplifi- cation of the cDNA at 95°C for 15 min followed by 35 cycles of 94°C for 1 min (denaturing), 52–63°C for 1.5 min (annealing), and 72°C for 2 min (extension) followed by a 72°C final extension for 20 min. The RT-PCR products were run on a 2% agarose gel with ethidium bromide and visualized with Eagle Eye II (Stratagene).

RESULTS

Figure 1 demonstrates the specificity of anti-α-hENaC and anti-β-hENaC antibodies. Figure 1A shows that anti-α-hENaC recognized a glycosylated form of the protein at 100 kDa in MDCK cells stably expressing αβγ-rENaC but not in untransfected cells (Fig. 1B) by Western blotting. Figure 1C is the autoradiograph of in vitro translated α-rENaC, and Fig. 1D is the anti-α-hENaC immunoprecipitated protein of ~75–80 kDa. Figure 1, E and F, represents the Western blots of expressing and nonexpressing MDCK cells probed with anti-β-ENaC, which recognized a protein of 100 kDa in expressing cells. Figure 1G is the auto-

<table>
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<th>Subunit</th>
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<td>13–397</td>
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ASIC, acid-sensing ion channel; ENaC, epithelial Na⁺ channel; h, human sequence; r, rabbit sequence.
radiograph of in vitro translated β-rENaC, and the immunoprecipitate of the same protein with anti-β-hENaC antibody is shown in Fig. 1H. Anti-β-hENaC also pulled down a protein of ~75–80 kDa.

Figure 2 includes representative images of αβγ-rENaC MDCK cells immunolabeled with anti-α-hENaC (Fig. 2A), anti-β-hENaC (Fig. 2B), and normal rabbit IgG as a control (Fig. 2C). The anti-ENaC antibodies showed brighter labeling at the cell membrane with some filamentous staining. Normal rabbit IgG did not specifically label these cells.

Figure 3 shows the Western blot of U251-MG cells probed with anti-ASIC1 (Fig. 3A), normal rabbit IgG (Fig. 3B), and the secondary antibody only (Fig. 3C). Anti-ASIC1 recognized a protein of ~71 kDa that is likely the glycosylated form of ASIC1. This protein was not recognized by the IgG and secondary antibodies.

Figure 4 depicts the immunohistochemical labeling with anti-α-hENaC and anti-β-ENaC in retinal sections. Localization of α-ENaC was specific for Müller cells (Fig. 4A), whereas β-ENaC localization occurred at the ganglion cell layer (GCL) and INL (Fig. 4B). Normal serum did not show specific labeling (Fig. 4C). Figure 4D is a section stained with QuickStain for comparison.

The pattern of labeling with anti-α-hENaC is shown in Fig. 5. Labeling was specific for Müller cells and was concentrated in, but not restricted to, the inner half of the cell including the cell body and the endfoot processes (Fig. 5A). Vimentin localized to the entire cell including the thin processes that extend to the outer limiting membrane (Fig. 5B). As a result, the overlay image clearly demonstrates the spatial localization of α-ENaC (Fig. 5C). These findings were consistent regardless of the type of fixation. After ~1 wk in culture, Müller cells in poly-L-ornithine-coated dishes developed a fibroblast-like morphology. Some cells appeared multinucleate and extended processes to neighboring cells. Despite this altered phenotype, these cells dis-
played α-hENaC (Fig. 5D) and vimentin (Fig. 5E) immunoreactivity. Figure 5F shows the overlay image. Vimentin staining appeared filamentous with nuclear staining, and α-hENaC staining also appeared filamentous with brighter staining around the cell perimeter. Some cell nuclei were also stained with this antibody.

Anti-β-hENaC showed clear localization in the GCL and diffuse labeling in the INL. To identify the specific cell types that express β-ENaC, markers including calbindin, PKC, TH, ChAT, and calretinin were used for double labeling. Anti-β-hENaC colocalized with the amacrine and ganglion cell markers predominantly in the cell bodies but not their processes (Fig. 6, C–E). Double labeling was sometimes seen with calbindin for on-cone bipolar cells and horizontal cells and with PKC for rod bipolar cells (Fig. 6, A and B). Interestingly, ganglion cell and amacrine cell labeling was consistently brighter than bipolar cell labeling.

Figure 7 shows that on Western blots of total retinal protein, anti-α-hENaC specifically recognized proteins at ~75 and 45 kDa (Fig. 7A), anti-β-hENaC recognized proteins at 123, 83, 73, 45, and 40 kDa (Fig. 7B), and anti-ASIC1 recognized 120-, 70-, 43-, and 36-kDa proteins (Fig. 7C). None of these proteins was recognized by normal rabbit IgG (Fig. 7D) or the secondary antibody (Fig. 7E).

RT-PCR analysis demonstrated the presence of α-, β-, γ-, and δ-ENaC and ASIC1–4. Primers designed from human sequences were generally successful and gave the anticipated products. However, the primers for α-ENaC and ASIC3 produced additional nonspecific products whereas human primers for β-ENaC gave no product. As a result, we designed rabbit-specific primers for β-ENaC. These primers produced only the predicted product. Figure 8 shows the agarose gel of the RT-PCR products for each subunit. Direct DNA sequencing of the cloned RT-PCR products for α-, β-, γ-, and δ-ENaC and ASIC1–4 confirmed their identities.

Whole cell patch clamp was used to demonstrate directly amiloride-sensitive Na+ current consistent with Na+ current known to be conducted by ENaC (4). In the basal state, cells exhibited “ragged” inward currents that were blocked after 100 μM amiloride perfusion. Figure 9 shows a representative recording with an inward current that was blocked by 100 μM amiloride.

DISCUSSION

Our study provides direct evidence for the existence of amiloride-sensitive Na+ channels in both neurons and glia of the rabbit retina. At the RNA level, all known ENaC and ASIC subunits appear to be expressed in these retinas. Unique antibodies against α- and β-ENaC show distinct localization. We also presented several lines of evidence demonstrating the specificity of our anti-α-hENaC, anti-β-hENaC, and anti-ASIC1 antibodies. Furthermore, we present the first physiological data demonstrating the presence of an amiloride-sensitive current in Müller glia.

A previous study reported immunohistochemical evidence for the expression of α-ENaC in cultured rat Müller cells (17). Our anti-α-hENaC antibody also localized to rabbit Müller cells. In frozen sections, the most prominent staining was at the inner portion of the cells and their endfeet. This pattern of ion channel expression is reminiscent of voltage-gated, inward-rectifying K+ channels whose function is to siphon K+ from the extracellular retinal space to the vitreous (29). It may be that ENaC performs a similar function for Na+. In cultured cells, anti-α-ENaC staining appeared filamentous, which agrees with Golestanesh et al. (17). As in vivo, cells expressing α-ENaC also expressed vimentin and thus confirmed that they were appropriate for patch-clamp analysis. The colocalization with vimentin can be attributed to the association of ENaC with cytoskeletal proteins such as actin (3, 8, 17, 35). Our evidence suggests that Müller cells do not express an ENaC channel composed of α-, β-, and γ-subunits. Müller cells may, in fact, represent the first example of an α-ENaC homomer in vivo, or the channel composition may include the ASIC subunits or a novel subunit.

Our data differ from the immunolocalization patterns of α-ENaC reported in rat (28). Possible reasons...
Fig. 6. Sections of rabbit retina showing β-ENaC localization in specific neurons of the GCL and INL by double labeling with antibodies against neuronal markers. Single arrows point to cells expressing β-ENaC and markers for cone on-bipolar cells (A), rod bipolar cells (B), dopaminergic amacrine cells (C), cholinergic cells (D), and ganglion cells (E; double arrows indicate AII amacrine cells). 1: labeled with anti-β-hENaC. 2: labeled with antibodies for neuronal markers. 3: overlay images.
for this include differences in the α-ENaC antibodies, species differences, or tissue fixation. We demonstrated that our antibodies were specific. However, species differences probably do not account for the conflicting findings, either. We screened several different tissue fixation protocols, and we believe that paraformaldehyde gave the best labeling that was above normal IgG background. Because fixations affect the availability of epitopes for antibodies, this is the most likely reason for the observed differences in localization patterns. We observed faint labeling with picric acid fixation, although it was not appreciably above normal rabbit IgG background.

The δ-subunit may serve α-like functions in the retina because it is found in brain and the characterization of this subunit revealed biochemical and physiological similarities with α-ENaC but not β- or γ-ENaC (38). The α-subunit is important in forming the channel pore (32), anchoring the channel complex to the cytoskeleton (12), and conferring H⁺ sensitivity (9).

Anti-β-ENaC labeling was restricted to cell bodies in the GCL and INL. We observed colocalization of β-ENaC with several amacrine cell markers and a ganglion cell marker, and β-ENaC also appeared to be expressed in rod and cone bipolar cells. Although we observed brighter labeling of amacrine cells and ganglion cells than bipolar cells, the significance of this is not clear.

Western blotting suggested the presence of α-ENaC, β-ENaC, and ASIC1 proteins in the rabbit retina. Antibodies against these subunits recognized proteins of
molecular masses consistent with theoretical calculations and published data (34). Theoretical molecular masses for the unglycosylated proteins are 70–75 kDa for \( \alpha \)- and \( \beta \)-ENaC and 60 kDa for ASIC1. The higher-molecular-mass bands on the blots represent glycosylated forms and aggregated proteins. Degradation products are represented by the lower-molecular-mass bands.

RT-PCR analysis demonstrated the presence of all known ENaC and ASIC subunits at the RNA level in the rabbit retina. Subsequent DNA sequencing confirmed their identities.

Whole cell patch-clamp data from cultured Müller cells showed ragged inward currents at hyperpolarizing potentials. This is a characteristic feature of ENaC in that they tend to exhibit cooperative opening and closing (5). Inward currents were completely blocked with high concentrations of amiloride; accordingly, this is consistent with the expression of an ASIC subunit in addition to \( \alpha \)-ENaC because the \( IC_{50} \) is higher for ASIC channels (10–20 \( \mu M \)) than for ENaCs (0.1–1 \( \mu M \)).

Despite extensive characterization of the ENaC/DEG family of channels in the brain, a specific physiological role has not yet been established. It is plausible that some proposed functions for these proteins in brain may also apply to the retina. Electrophysiology indicates that the amiloride-sensitive Na\(^{+}\) current is not voltage dependent. Therefore, it is unlikely that these channels play a role in action potential generation. Osmotic pressure has been shown to regulate \( \alpha \)-ENaC in that they tend to exhibit cooperative opening and closing (5). Inward currents were completely blocked with high concentrations of amiloride; accordingly, this is consistent with the expression of an ASIC subunit in addition to \( \alpha \)-ENaC because the \( IC_{50} \) is higher for ASIC channels (10–20 \( \mu M \)) than for ENaCs (0.1–1 \( \mu M \)).

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