Acta Medica Okayama

Volume 52, *Issue* 5 1998 *Article* 7

OCTOBER 1998

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Abstract

To study the expression of the amiloride-sensitive sodium channel, a putative mechano-receptor in the rat eye, reverse transcriptase-polymerase chain reaction and in situ hybridization were done. The gene for the alpha subunit of the amiloride-sensitive sodium channel was shown by polymerase chain reaction to be expressed in mRNA isolated from the whole eye tissue. In situ hybridization demonstrated that the gene was expressed in basal layers of the corneal and conjunctival epithelium, ciliary epithelial cells, lens epithelial cells at the equator, retinal and iris pigment epithelial cells, ganglion cells and cells in the inner and outer nuclear layers of the retina. The results suggest that the amiloride-sensitive sodium channel plays a role in maintaining sodium balance as well as in possible mechanosensation in these ocular tissues.

KEYWORDS: amiloride-sensitive sodium channel, eye, insitu hybridzation, mechanosensation, polymerase chain reaction

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*PMID: 9810438 [PubMed - indexed for MEDLINE]

ACTA MED OKAYAMA 1998; 52(5): 279-283

Brief Note

Expression of Amiloride-Sensitive Sodium Channel in Rat Eye

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To study the expression of the amiloride-sensitive sodium channel, a putative mechanoreceptor in the rat eye, reverse transcriptase-polymerase chain reaction and *in situ* hybridization were done. The gene for the alpha subunit of the amiloride-sensitive sodium channel was shown by polymerase chain reaction to be expressed in mRNA isolated from the whole eye tissue. *In situ* hybridization demonstrated that the gene was expressed in basal layers of the corneal and conjunctival epithelium, ciliary epithelial cells, lens epithelial cells at the equator, retinal and iris pigment epithelial cells, ganglion cells and cells in the inner and outer nuclear layers of the retina. The results suggest that the amiloride-sensitive sodium channel plays a role in maintaining sodium balance as well as in possible mechanosensation in these ocular tissues.

Key words: amiloride-sensitive sodium channel, eye, *in situ* hybridization, mechanosensation, polymerase chain reaction

Stretch-activated ion channels (mechanoreceptors) have been identified electrophysiologically in various tissues (1, 2). In the eye, corneal (3, 4) and lens (5) epithelial cells have been shown to contain stretch-activated channels. In addition, I previously demonstrated that some human trabecular cells in culture showed a transient rise or oscillation of intracellular calcium in response to the elevation of hydraulic pressure (6). Recently, genes encoding three subunits (alpha, beta, and gamma subunit) of a rat amiloride-sensitive sodium channel have been cloned and found to share significant sequence similarity with Caenorhabditis elegans genes involved in sensory touch transduction, leading to the notion that this channel is a mechanoreceptor (7-11).

The amiloride-sensitive sodium channel plays a major role in regulation of the volume and sodium balance of body fluids. Mutations in genes for subunits of the amiloride-sensitive sodium channel have been found in such human diseases as Liddle syndrome (12, 13) and pseudohypoaldosteronism type 1 (14, 15). Mutations resulting in hyperfunction of the channel are associated with hypertension as seen in Liddle syndrome (12, 13), while mutations resulting in its hypofunction are associated with salt wasting syndrome (14, 15). A transgenic mouse, lacking the alpha subunit of the channel, showed defective neonatal lung liquid clearance and died younger than usual (16).

In this study, the expression of the amiloride-sensitive sodium channel was studied in the rat eye to understand its role in regulation of sodium balance as well as possible mechanosensation in eye tissues.

Materials and Methods

Polymerase chain reaction. After rats were anesthetized with ether, the eyes were enucleated, and mRNA was purified from the whole eye with a Fast Track mRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized with AMV reverse transcriptase, and polymerase chain reaction was done with Taq polymerase (TaKaRa RNA PCR Kit, Takara Shuzo, Kyoto, Japan) using primers (7, 8), 5'-ATGCTGGACCACACCAGAGC-3' and 5'-AGTCCTTCCGGTCCACTTGG-3' in a condition of denaturation at 94°C for one minute, annealing at 65°C for one minute, and elongation at 72°C for one minute. The two primers were designed to amplify the initial third of the coding region

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with a length of 745 base pairs for the alpha subunit of the amiloride-sensitive sodium channel. The sequence of the primers for the alpha subunit did not show homology with that of the beta and gamma subunits (7–9).

In situ hybridization. Digoxigenin-labeled antisense and sense RNA probes were synthesized with a DIG RNA Labeling Kit (SP6/T7) (Boehringer Mannheim Japan, Tokyo). Briefly, Plasmids (8) containing cDNA for the alpha subunit of the rat amiloride-sensitive sodium channel (pEXO RCNaCh1) were linearized by digestion with EcoRI or XhoI. Antisense and sense RNA were synthesized with ribonucleotidyl triphosphates including digoxigenin-conjugated uridine triphosphate from the EcoRI-digested plasmid and the XhoI-digested plasmid as a template by SP6 RNA polymerase and T7 RNA polymerase, respectively, for 2 h at 37°C. After digestion of template plasmids by RNase-free DNase I, RNA probes were concentrated by ethanol precipitation and then exposed to alkali treatment.

Male rats at 6 weeks of age were anesthetized with ether and perfused from the left ventricle of the heart with 4 % paraformaldehyde in phosphate-buffered saline (PBS: 100 mM NaCl, 10 mM sodium phosphate, pH 7.4). Eyes were enucleated, fixed further, and embedded in paraffin. Paraffin sections were cut, deparaffinized with xylene and rehydrated with a graded series of alcohol. After quenching with glycine and acetylation, sections were prehybridized in 50 % formamide and 2 × SSC (1 × SSC: 150 mM NaCl, 15 mM sodium citrate) at 42°C for 30 min, and hybridized with a 1 μg/mL digoxigenin-labeled RNA probe in 50 % formamide, 2 × SSC, 1 mg/mL tRNA, 1 mg/mL sonicated salmon sperm DNA, 1 mg/mL bovine serum albumin, 10 % dextran sulfate, and 10 mM dithiothreitol at 42°C for 16 h. Sections were washed with 50 % formamide and 2 × SSC at 42°C for 60 min, treated with ribonuclease, and washed with 0.1 × SSC (17).

Signals were detected with a DIG Nucleic Acid Detection Kit (Boehringer Mannheim). Briefly, the sections were incubated with a blocking reagent and then with anti-digoxigenin Fab-fragments conjugated with alkaline phosphatase for 30 min. After washing, color was developed with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt.

Results and Discussion

A single band of about 700 base pairs was generated by reverse transcriptase-polymerase chain reaction using mRNA from the whole eye (Fig. 1). The band was confirmed by sequencing to be derived from alpha subunit of amiloride-sensitive sodium channel.

In situ hybridization, using as a probe the whole coding sequence for the alpha subunit of the amiloride-sensitive sodium channel, demonstrated that the gene was expressed interruptively in some basal layer cells of the corneal and conjunctival epithelium, in contrast with its continuous expression in the ciliary epithelium (Fig. 2). Continuous expression of the gene was also found in retinal and iris pigment epithelial cells (Fig. 3). The expression in the lens was restricted to the lens epithelial cells at the equator where ciliary zonules were inserted (Fig. 3). The expression in the sensory retina was localized to ganglion cells, the inner and outer nuclear layers (Fig. 3).

It should be noted that only basal layers of the corneal and conjunctival epithelium showed interrupted, non-continous, expression of the gene for the amiloride-sensitive sodium channel. Some cells in these epithelia may have a specific role in maintaining sodium balance in the cornea and conjunctiva as well as in possible mechanosensation. Stretch-activated channels, which have been characterized electrophysiologically in corneal epithelial cells, were found to be either potassium-selective or non-selective (3, 4), and therefore different from this amiloride-sensitive sodium channel. The expression of the gene in the ciliary epithelium might play a role in aqueous production and might also be involved in possible mechanosensation of intraocular pressure for feedback regulation of aqueous production.

It is particularly interesting to note that the expression in the lens is restricted to lens epithelial cells at the equator. Since ciliary zonules are present at the equator of the lens and originating from the basement membrane of lens epithelial cells, these cells at the equator would receive the greatest degree of stretching force during accommodation. Possible mechanosensation by these epithelial cells at the equator of the lens might play a role in maintaining lens homeostasis during accommodation. A stretch-activated channel which has been found electrophysiologically in lens epithelium is mainly selective to potassium (5) and different from the amiloride-sensitive sodium channel.

The expression of the channel by the retina seems to indicate that retinal neurons play a role in mechanosensation, but the meaning of this finding remains unclear. The amiloride-sensitive sodium channel has been implicated in the formation of the neural tube during development by

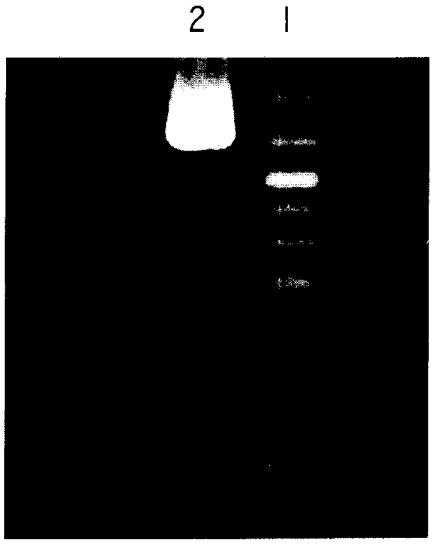

Fig. 1 Reverse transcriptase-polymerase chain reaction, generating a band of about 700 base pairs from mRNA isolated from the whole eye tissue with primers for the alpha subunit of the amiloride-sensitive sodium channel (lane 2). DNA markers (top to bottom: 1000, 700, 525, 500, 400, 300, 200, 100 and 50 base pairs: BioMarker Low, BioVentures, Murfreesboro, TN, USA) are given in lane 1.

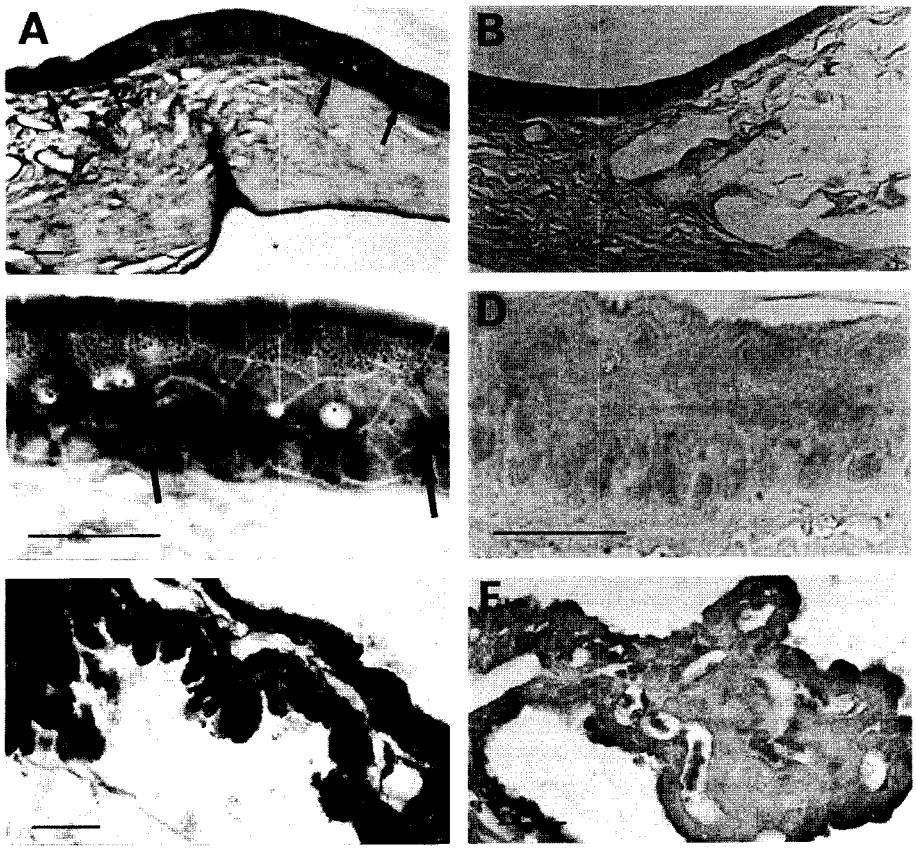

Fig. 2 *In situ* hybridization using as a probe the whole coding sequence of the alpha subunit of the amiloride-sensitive sodium channel in the rat eye. The gene is expressed interruptively in basal layers of the corneal (arrows in **A**, **C**) and conjunctival epithelium (arrows in **A**), and continuously in the ciliary epithelium (**E**). Hybridization with antisense RNA probe is shown in **A**, **C** and **E**, while that with sense RNA probe is shown in **B**, **D** and **F**. Bar = 20 μm.

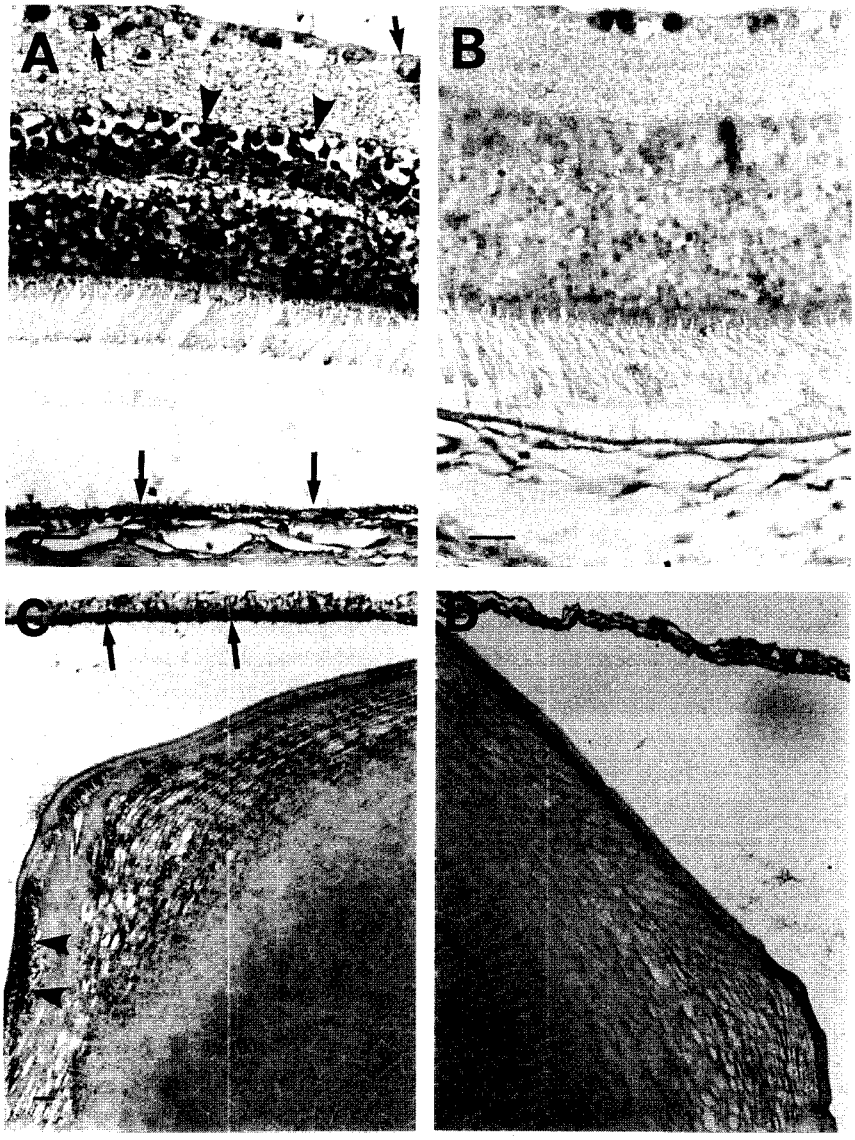

Fig. 3 *In situ* hybridization using as a probe the whole coding sequence of the alpha subunit of the amiloride-sensitive sodium channel in the rat eye. The gene expression in the retina is visible in ganglion cells (small arrows in **A**), inner (arrowheads in **A**) and outer (asterisk in **A**) nuclear layers, and pigment epithelial cells (large arrows in **A**). The gene is also expressed in lens epithelial cells only at the equator (arrowheads in **C**), and iris pigment epithelial cells (arrows in **C**). Hybridization with antisense RNA probe is shown in **A** and **C**, while that with sense RNA probe is shown in **B** and **D**. Bar = 20 μm.

regulating the volume of the neural tube (18). In the same way, the expression of the gene in the retina may be involved in volume regulation of the vitreous cavity.

The trabecular tissue did not show notable expression of the gene although trabecular cells are known to respond to mechanical stimuli such as hydraulic pressure (6) and cyclic mechanical stretching (19, 20). This may be due to a low level of the expression, or the trabecular tissue may have a different type of mechanoreceptor.

Acknowledgments. This work was supported in part by a Grant-in-Aid for Scientific Research (B2-09470380) from the Ministry of Education, Science, Sports and Culture of Japan, and also by the 1996 Suda Memorial Fund for Glaucoma Research. The author thanks Drs. Pascal Barbry and Michel Lazdunski of the Institut de Pharmacologie Moleculaire et Cellulaire, Centre National de la Recherche Scientifique, Valbonne, France for kindly supplying a plasmid pEXO RCNaCh1 (8).

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Received March 23, 1998; accepted July 17, 1998.