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Defective Secretion of Saliva in Transgenic Mice Lacking Aquaporin-5 Water Channels*

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Tonghui Ma, Yualin Song, Annemarie Gillespie, Elaine J. Carlson, Charles J. Epstein, and A. S. Verkman‡

From the Departments of Medicine, Physiology, and Pediatrics, Cardiovascular Research Institute, University of California, San Francisco, California 94143-0521

Aquaporin-5 (AQP5) is a water-selective transporting protein expressed in epithelial cells of serous acini in salivary gland. We generated AQP5 null mice by targeted gene disruption. The genotype distribution from intercross of founder AQP5 heterozygous mice was 70:69:29 wild-type:heterozygote:knockout, indicating impaired prenatal survival of the null mice. The knockout mice had grossly normal appearance, but grew ~20% slower than litter-matched wild-type mice when placed on solid food after weaning. Pilocarpine-stimulated saliva production was reduced by more than 60% in AQP5 knockout mice. Compared with the saliva from wild-type mice, the saliva from knockout mice was hypertonic (420 mosM) and dramatically more viscous. Amylase and protein secretion, functions of salivary mucous cells, were not affected by AQP5 deletion. Water channels AQP1 and AQP4 have also been localized to salivary gland; however, pilocarpine stimulation studies showed no defect in the volume or composition of saliva in AQP1 and AQP4 knockout mice. These results implicate a key role for AQP5 in saliva fluid secretion and provide direct evidence that high epithelial cell membrane water permeability is required for active, near-isosmolar fluid transport.

The family of molecular water channels (aquaporins) numbers 10 in mammals and many more in plants and lower organisms. There has been considerable recent interest in the role of aquaporins in mammalian physiology and disease mechanisms. In humans, mutation of the vasopressin-regulated water channel of kidney collecting, AQP2,[1] causes hereditary nephrogenic diabetes insipidus in which patients are unable to concentrate their urine (1). Recent phenotype characterization of transgenic knockout mice lacking AQP1 and AQP4 has been very informative in defining the roles of these water channels in the urinary concentrating mechanism, lung fluid transport, and gastrointestinal physiology (2–6). However the phenotype studies indicated that the tissue expression of an aquaporin does not ensure its functional significance.

AQP5 is a water channel with a unique tissue expression pattern (7). Immunocytochemical studies from several laboratories showed AQP5 expression in the apical membranes of serous acinar cells in salivary and lacrimal glands, type I alveolar epithelial cells, and surface corneal epithelial cells (8–11). AQP5 appears to function as an unregulated water-selective channel with comparable intrinsic water permeability to AQP1 (12). The human AQP5 gene contains 4 exons with exon-intron boundaries at identical locations to those several other aquaporins (13); the genes for AQP5, AQP2, and AQP6 are clustered in a small 27-kb region at chromosome locus 12q13 (14). It was proposed that AQP5 plays an important role in glandular secretions of saliva and tears and that abnormalities in AQP5 might occur in some forms of Sjogren's syndrome (15, 16). Aquaporin gene delivery to salivary gland has been proposed to increase fluid secretion (15). However, these possibilities are based on the unproven assumption that AQP5 is a major pathway for water movement in salivary gland and that active near-isosmolar fluid secretion across acinar cells requires a high apical cell membrane water permeability.

The purpose of this study was to define the involvement of AQP5 in saliva secretion, as well as that of AQP1 and AQP4, water channels expressed in salivary gland capillaries and ducts (17, 18). Phenotype studies were done on AQP5 null mice generated by targeted gene disruption, as well as on AQP1 and AQP4 null mice. The principal finding was that AQP5 deletion is associated with production of a low volume hypertonic viscous saliva, providing direct evidence for a role of AQP5 in near-isosmolar fluid secretion in salivary gland.

MATERIALS AND METHODS

Generation of AQP5 Null Mice—The cDNA encoding mouse AQP5 was isolated from salivary gland cDNA by PCR based on homology to rat AQP5. The structure of the mouse AQP5 gene was analyzed by PCR amplification of exon-intron-exon fragments using C57BL6/J mouse genomic DNA as template. A targeting vector was constructed using a 1.4-kb fragment of genomic DNA containing partial exon 1, intron 1, and partial exon 2 (left arm) and a 4.2-kb fragment containing exon 4 and downstream genomic DNA (right arm). The left and right genomic fragments (flanking a 1.8-kb PolIIneobpA cassette) were PCR-amplified, and a PGK-tk cassette was inserted upstream for negative selection. The vector was linearized at a unique downstream *Not*I site and electroporated into CB1–4 embryonic stem (ES) cells. Transfected ES cells were selected with G418 and FIAU for 7 days, yielding seven targeted clones out of 286 doubly resistant colonies upon PCR screening using a *neo*-specific antisense primer and an AQP5 gene-specific sense primer located 30 base pairs upstream of the targeting region. Homologous recombination was confirmed by Southern hybridization in which 10 μg of genomic DNAs were digested with *Xba*I, electrophoresed, transferred to a Nylon+ membrane (Amersham Pharmacia Biotech), and hybridized with a 0.7-kb genomic fragment as indicated in Fig. 1*A*. ES cells were injected into PC 2.5 day 8 cell morula stage CD1 zygotes, cultured overnight to blastocysts, and transferred to pseudopregnant B6D2 females. Offspring from breeding of chimeras and wild-type mice were genotyped by PCR followed by Southern blot analysis as described above. Heterozygous founder mice were intercrossed to produce homozygous AQP5 knockout mice.

Northern Blot Analysis—RNA from submandibular gland was isolated using TRIzol reagent (Life Technologies, Inc.). RNAs (10 μg/lane) were resolved on a 1.2% formaldehyde-agarose denaturing gel, transferred to a Nylon+ membrane (Amersham Pharmacia Biotech), and hybridized at high stringency with a ^{32}P-labeled probe corresponding to

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‡ To whom correspondence should be addressed: 1246 Health Sciences East Tower, Cardiovascular Research Institute, University of California, San Francisco, CA 94143-0521. Tel.: 415-476-8530; Fax: 415-665-3847; E-mail: verkman@itsa.ucsf.edu; http://www.ucsf.edu/verklab.

[1] The abbreviations used are: AQP, aquaporin; kb, kilobase(s); PCR, polymerase chain reaction.

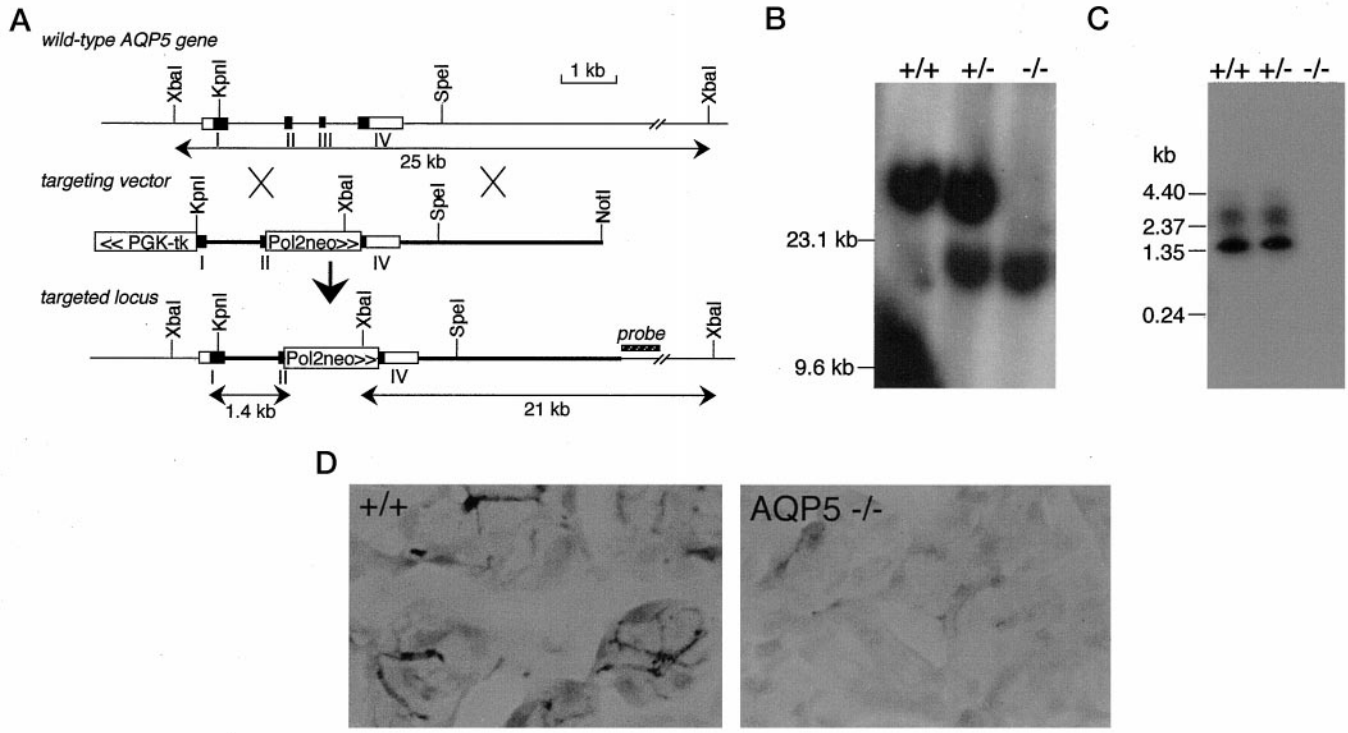

FIG. 1. *A*, targeting strategy for AQP5 gene interruption. Homologous recombination results in replacement of exon 3, part of exons 2 and 4, and introns 2 and 3 by a 1.8-kb polII-neo-selectable marker. The probe used for Southern blot analysis is indicated (labeled "*probe*") and the 1.4-kb amplified region for PCR analysis is shown. *B*, Southern blot of mouse liver genomic DNA digested with *Xba*I and probed as indicated in *A*. *C*, Northern blot of salivary gland probed with the mouse AQP5 coding sequence. *D*, immunoperoxidase localization of AQP5 in salivary gland of wild-type (*left*) and AQP5 knockout (*right*) mice.

the mouse AQP5 cDNA coding sequence.

Immunocytochemistry—A polyclonal antibody was raised in rabbits against a synthetic peptide (NH_2-CEEDEDHREERKK-COOH) corresponding to the predicted C terminus of mouse AQP5. Immunoperoxidase localization of AQP5 protein in fixed frozen section of salivary gland was done using a 1:500 dilution of serum as described previously (18).

Saliva Collections—Mice were anesthetized using intraperitoneal nembutol (50 mg/kg). Saliva production was stimulated by subcutaneous injection of pilocarpine (80 mg/kg) as described previously (19). Salivation generally occurred in less than 20 s. Saliva was collected in two 5-min intervals using a preweighed suction apparatus. Mice were positioned on their side with head slightly down to facilitate suctioning every 15–30 s. The mice were sacrificed after saliva collection, and tissues were harvested as needed. The investigator was blinded to mouse genotype for saliva collections.

Analysis of Saliva—At the end of saliva collection, the pH was determined using pH paper and the total amount of saliva by gravimetry. Saliva osmolality, and concentrations of sodium, potassium, and chloride were determined by the University of California San Francisco clinical chemistry laboratory.

RESULTS AND DISCUSSION

Analysis of the mouse AQP5 gene indicated 4 exons separated by 3 introns (lengths 1.3, 0.6, and 0.8 kb) in the coding region. Exon-intron boundaries were located at residues 122, 176, and 204 in the AQP5 coding sequence, as was reported for the human AQP5 gene (13) and for the mouse AQP2 (20) and AQP6 (21) genes. Genomic Southern blot analysis showed a single copy mouse AQP5 gene per haploid genome. Fig. 1*A* shows the targeting vector for targeted AQP5 gene deletion. Part of exon 2, exon 3, part of exon 4, and introns 1 and 2 were replaced by a *neo* cassette to prevent the expression of functional AQP5 in the null mice.

Fig. 1*B* shows Southern blot analysis of liver DNA from a wild-type, heterozygote, and AQP5 knockout mice. The band at 21 kb corresponds to the modified gene locus. Fig. 1*C* shows a Northern blot analysis of salivary gland mRNA. A single transcript of ~1.6 kb was observed in wild-type and heterozygous mice. No full-length transcript was seen in the AQP5 knockout mice. By immunocytochemistry, AQP5 was localized at the apical pole of acinar cells in the salivary gland of wild-type mice (Fig. 1*D*, *left*), in agreement with results in rat (10, 11, 22), with no detectable AQP5 protein in the knockout mice (Fig. 1*D*, *right*).

The AQP5 null mice were grossly normal in appearance and general activity, except for mild growth retardation seen within the first weeks after weaning (Fig. 2*A*). We believe that slowed growth of AQP5 null mice when placed on solid food is a consequence of defective saliva production (see below), because no differences in growth were found when mice were placed on a Peptamine liquid diet after weaning. Genotype analysis of 168 offspring from intercross of founder AQP5 heterozygotes showed a 70:69:29 distribution of wild-type:heterozygote:knockout mice, indicating decreased prenatal survival of AQP5 null mice. For comparison, an ~1:2:1 genotype ratio was found for intercross of AQP4 heterozygous mice (2), whereas significantly fewer mice of the AQP1 null genotype were found from intercross of AQP1 heterozygous mice (3).

A method was developed to reproducibly measure total saliva secretion, which contains contributions from submandibular, sublingual, and parotid glands, each of which expresses AQP5 in serous acinar cells (22). Saliva secretion was induced in anesthetized mice by pilocarpine and collected using a mini-suction apparatus based on a recent design (23). In wild-type mice, marked salivation was observed within 20 s after pilocarpine injection, and substantial amounts of clear, nonviscous fluid were collected. The amount of collected fluid was reproducible: mean and S.D. values were 228 ± 21 mg and 154 ± 17 mg for the first and second 5 min periods after pilocarpine injection, respectively. Less than 5 mg of fluid could be collected over 5 min in anesthetized mice that did not receive pilocarpine.

The saliva collected from every pilocarpine-stimulated AQP5

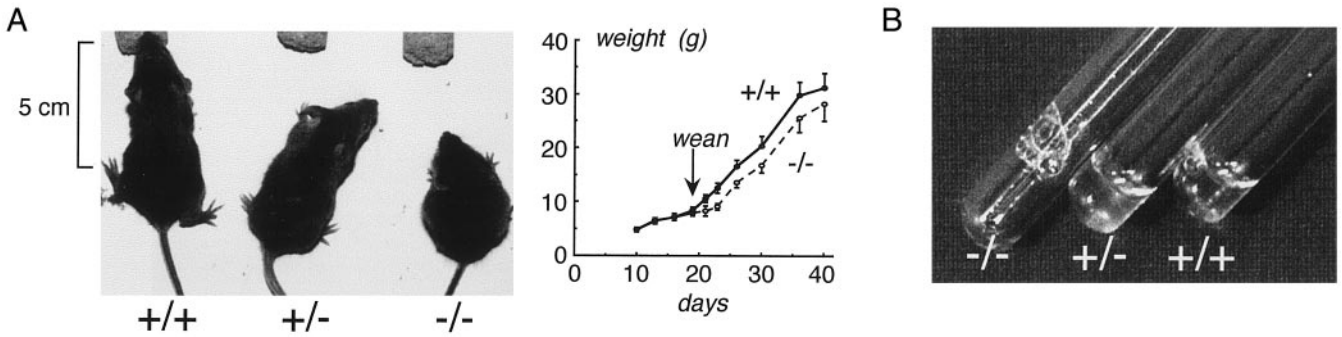

FIG. 2. **Growth and saliva secretion in AQP5 null mice.** *A*: *left*, photograph of a wild-type, heterozygous, and AQP5 null mouse at 3 weeks of age, 4 days after weaning; *right*, mouse growth. *B*, saliva collected from mice of indicated genotype over the first 5 min after pilocarpine stimulation.

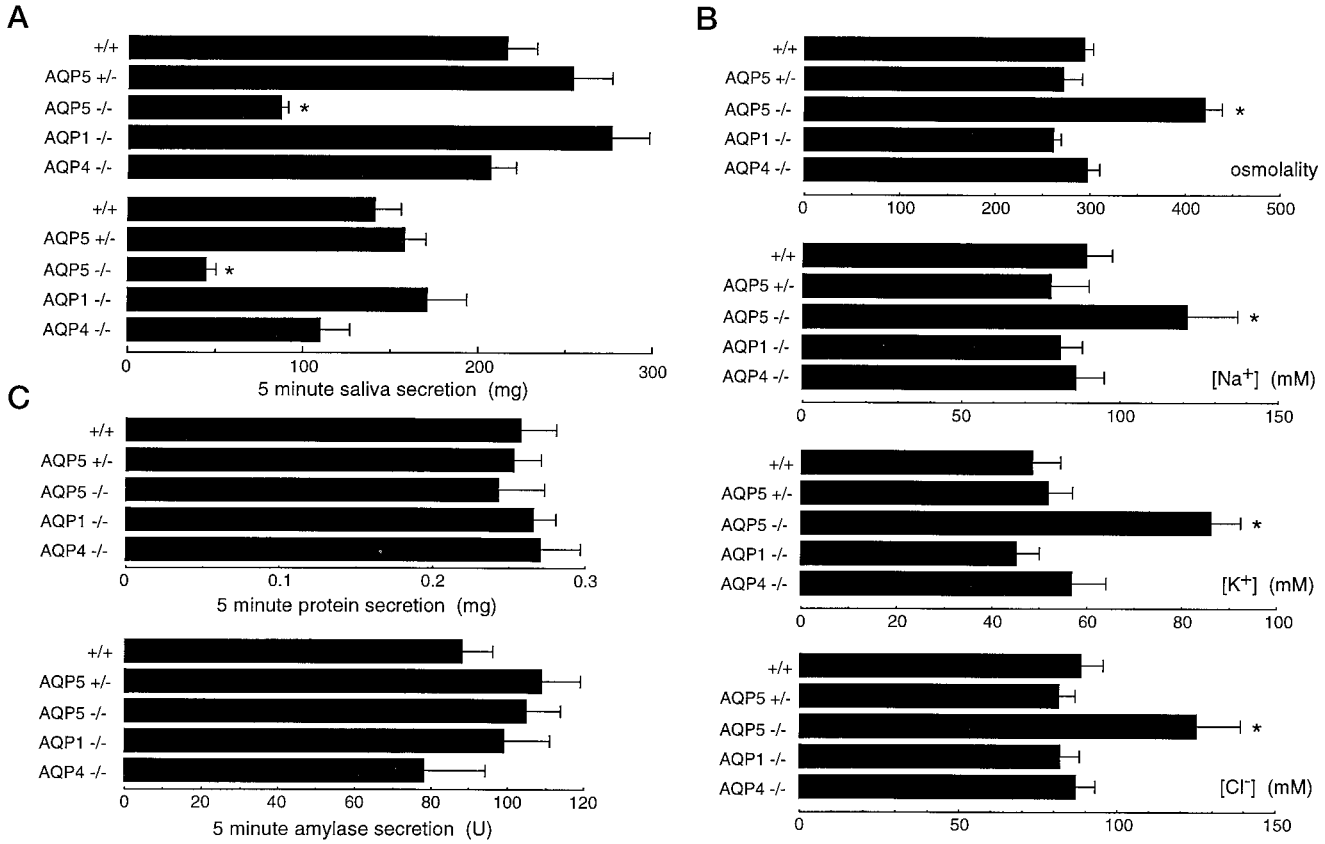

FIG. 3. **Effect of aquaporin deletion on saliva composition.** Data are shown as mean ± S.E. for collections made on four to six mice in each group. *A*, total amount (weight) of saliva collected over the first and second 5-min periods after pilocarpine injection. *B*, osmolality and electrolyte concentrations of saliva collected over the first 5 min. *C*, total protein content and amylase activity of saliva collected over the first 5 min.

null mouse was remarkably viscous and tenacious and of lower volume than that collected from wild-type and heterozygous mice (Fig. 2*C*). Fig. 3*A* summarizes the amount of saliva collected in the first and second 5-min periods from wild-type mice, AQP5 heterozygous and null mice, and AQP1 and AQP4 null mice. The reduced production of saliva in AQP5 null mice indicates defective serous cell function. Surprisingly, deletion of AQP1 in the salivary microvascular endothelia did not affect saliva production, which contrasts with the marked effects found when AQP1 is deleted in lung and renal microvessels (3, 6).

The collected saliva from wild-type and AQP5 null mice was analyzed for osmolality, electrolyte composition, protein content, amylase activity, and pH. Fig. 3*B* shows that the saliva from AQP5 null mice was hypertonic, with significantly higher osmolality than saliva from the wild-type mice and the AQP1 and AQP4 null mice. The concentrations of Na^+, K^+, and Cl^- were also higher. Total protein secretion and amylase activity were not affected by deletion of AQP5, AQP1, or AQP4 (Fig. 3*C*). Saliva pH measured immediately after collection (before HCO_3 loss) was in the range 9.0–10.0 for all mice.

The abnormal saliva volume, osmolality, and electrolyte content in AQP5 null mice implicates the involvement of AQP5 in transcellular fluid secretion across serous-type acinar cells. Serous acinar cells contain multiple salt-transporting proteins for fluid secretion, whereas mucous cells secrete proteinaceous materials, including amylase (24). The "primary saliva" produced by serous cells is modified during its transit through the salivary duct, where it is thought that salts are absorbed across a relatively water-impermeable ductal epithelium. The normal protein and amylase content of saliva from AQP5 null mice is consistent with the absence of AQP5 in mucous cells. Primary saliva should be near-isotonic, becoming progressively hypotonic during its passage through the salivary duct. The hyper-

tonic saliva from AQP5 null mice suggests that active acinar cell salt secretion into the gland lumen occurs without adequate amounts of water.

The decreased salivary gland fluid secretion in AQP5 null mice supports the paradigm that high epithelial cell membrane water permeability facilitates active near-isosmolar fluid secretion and absorption (reviewed in Ref. 25). Mechanistically, the small osmotic gradients produced by active salt pumping are able to drive water across highly water permeable cell membranes. Schnermann *et al.* (4) showed that AQP1 deletion in proximal tubule is associated with defective near-isosmolar fluid reabsorption. The data here show that high water permeability is needed for efficient near-isosmolar fluid transport in salivary gland. From the data of Schnermann *et al.* (4), ~0.5 μl/min of fluid are actively absorbed per cm^2 of proximal tubule surface (assuming a smooth surface); in the pilocarpine-stimulated mouse salivary gland, ~50 μl/min of saliva are secreted across an estimated 5 cm^2 of serous acinar cell apical membrane surface area (based on values given in Ref. 26 and assuming smooth surface), giving a high secretion rate of ~10 μl/min/cm^2. Water channels are thus likely to be required in tissues having rapid rates of active fluid transport, such as choroid plexus, ciliary body, and pancreatic acini.

The AQP5 null mice should have utility in defining the mechanisms of near-isosmolar fluid absorption in lung, tear secretion in lacrimal gland, and corneal transparency in eye. The mice are also a suitable host for delivery of aquaporin genes to increase saliva production. Last, the finding of salivary gland dysfunction in AQP5-deficient mice mandates the search for disease-causing mutations in human AQP5, as well as pharmacological modulators of AQP5, expression and function.

REFERENCES

1. Deen, P. M., Verkijk, M. A., Knoers, N. V., Wieringa, B., Monnens, L. A., Van Os, C. H., and Van Oost, B. A. (1994) *Science* **264,** 92–95
2. Ma, T., Yang, B., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (1997) *J. Clin. Invest.* **100,** 957–962
3. Ma, T., Yang, B., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (1998) *J. Biol. Chem.* **273,** 4296–4299
4. Schnermann, J., Chou, C. L., Ma, T., Traynor, T., Knepper, M. A., and Verkman, A. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9660–9664
5. Chou, C. L., Knepper, M. A., Van Hoek, A. N., Brown, D., Yang, B., Ma, T., and Verkman, A. S. (1999) *J. Clin. Invest.* **103,** 491–496
6. Bai, C., Fukuda, N., Song, Y., Ma, T., Matthay, M. A., and Verkman, A. S. (1999) *J. Clin. Invest.* **103,** 555–561
7. Raina, S., Preston, G. M., Guggino, W. B., and Agre, P. (1995) *J. Biol. Chem.* **270,** 1908–1912
8. Hamann, S., Zeuthen, T., LaCour, M., Nagelhus, E. A., Ottersen, O. P., Agre, P., and Nielsen, S. (1998) *Am. J. Physiol.* **274,** C1332–C1345
9. Ishida, N., Hirai, S. I., and Mita, S. (1997) *Biochem. Biophys. Res. Commun.* **238,** 891–895
10. Nielsen, S., King, L. S., Christensen, B. M., and Agre, P. (1997) *Am. J. Physiol.* **273,** C1549–C1561
11. Funaki, H., Yamamoto, F. H., Koyama, Y., Kondo, D., Yaoita, E., Kawasaki, K., Kobayashi, H., Sawaguchi, S., Abe, H., and Kihara, I. (1998) *Am. J. Physiol.* **275,** C1151–C1157
12. Yang, B., and Verkman, A. S. (1997) *J. Biol. Chem.* **272,** 16140–16146
13. Lee, M. D., Bhakta, K. Y., Raina, S., Yonescu, R., Griffin, C. A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Preston, G. M., and Agre, P. (1996) *J. Biol. Chem.* **271,** 8599–8604
14. Ma, T., Yang, B., Kuo, W. L., and Verkman, A. S. (1996) *Genomics* **35,** 543–550
15. Delporte, C., O'Connell, B. C., He, X., Lancaster, H. E., O'Connell, A. C., Agre, P., and Baum, R. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3268–3273
16. Delporte, C., Hoque, A. T., Kulakusky, J. A., Braddon, V. R., Goldsmith, C. M., Wellner, R. B., and Baum, B. J. (1998) *Biochem. Biophys. Res. Commun.* **246,** 584–588
17. Li, J., Nielsen, S., Dai, Y., Lazowski, K. W., Christensen, E. I., Tabak, L. A., and Baum, B. J. (1994) *Pfluegers Arch.* **428,** 455–460
18. Frigeri, A., Gropper, M., Umenishi, F., Kawashima, M., Brown, D., and Verkman, A. S. (1995) *J. Cell Sci.* **108,** 2993–3002
19. Marmary, Y., Fox, P. C., and Baum, B. J. (1987) *Comp. Biochem. Physiol.* **88A,** 307–310
20. Yang, B., Ma, T., Xu, Z., and Verkman, A. S. (1999) *Genomics* **57,** 79–83
21. Ma, T., Yang, B., Umenishi, F., and Verkman, A. S. (1997) *Genomics* **43,** 387–389
22. Matsuzaki, T., Suzuki, T., Koyama, H., Tanaka, S., and Takata, K. (1999) *Cell Tissue Res.* **295,** 513–521
23. Wolff, A., Begleiter, A., and Moskona, D. (1997) *J. Dent. Res.* **76,** 1782–1786
24. Nauntofte, B. (1992) *Am. J. Physiol.* **263,** G823–G837
25. Spring, K. R. (1998) *Annu. Rev. Physiol.* **60,** 105–119
26. Cook, D. I., and Young, J. A. (1989) in *Handbook of Physiology* (Schultz, S. G., Forte, J. G., and Rauner, B. B., eds) pp. 1–21, Oxford University Press,

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