Skeletal muscle function and water permeability in aquaporin-4 deficient mice

Baoxue Yang, Jean-Marc Verbavatz, Yuanlin Song, Geoffrey Manley, Wei-Ming Kao, Tonghui Ma, and A. S. Verkman. Skeletal muscle function and water permeability in aquaporin-4 deficient mice. Am J Physiol Cell Physiol 278: C1108–C1115, 2000.—It has been proposed that aquaporin-4 (AQP4), a water channel expressed in plasmalemma of skeletal muscle cells, is important in normal muscle physiology and in the pathophysiology of Duchenne's muscular dystrophy. To test this hypothesis, muscle water permeability and function were compared in wild-type and AQP4 knockout mice. Immunofluorescence and freeze-fracture electron microscopy showed AQP4 protein expression in plasmalemma of fast-twitch skeletal muscle fibers of wild-type mice. Osmotic water permeability was measured in microdissected muscle fibers from the extensor digitorum longus (EDL) and fractionated membrane vesicles from EDL homogenates. With the use of spatial-filtering microscopy to measure osmotically induced volume changes in EDL fibers, half times ($t_{1/2}$) for osmotic equilibration (7.5–8.5 s) were not affected by AQP4 deletion. Stopped-flow light-scattering measurements of osmotically induced volume changes in plasmalemma vesicles also showed no significant differences in water permeability. Similar water permeability, yet ~90% decreased AQP4 protein expression was found in EDL from mdx mice that lack dystrophin. Skeletal muscle function was measured by force generation in isolated EDL, treadmill performance time, and in vivo muscle swelling in response to water intoxication. No differences were found in EDL force generation after electrical stimulation [42 ± 2 (wild-type) vs. 41 ± 2 (knockout) g/s], treadmill performance time (22 vs. 26 min; 29 m/min, 13° incline), or muscle swelling (2.8 vs. 2.9% increased water content at 90 min after intraperitoneal water infusion). Together these results provide evidence against a significant role of AQP4 in skeletal muscle physiology in mice.

A recent paper reported that AQP4 in rat skeletal muscle is expressed more in fast than slow-twitch fibers and that apparent water permeability is higher in fast-twitch fibers (11). In addition, several groups reported decreased AQP4 expression in skeletal muscle of mdx mice (11, 16), a dystrophin-deficient mouse model of Duchenne's muscular dystrophy. It was proposed that decreased AQP4 expression may contribute to the pathophysiology and/or pathogenesis of hereditary muscular dystrophies. However, these lines of evidence do not prove a causal relationship between reduced AQP4 expression and abnormal skeletal muscle function.

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The study here utilized transgenic AQP4 deficient mice to test the hypothesis that AQP4 deletion results in decreased skeletal muscle water permeability and defective muscle function. With the use of a battery of in vitro and in vivo measurement strategies, we found that AQP4 deletion did not produce significant abnormalities in skeletal muscle water transport or function. In addition, no differences in water permeability were found in skeletal muscle from mdx mice. These results provide evidence against an important role of AQP4 in skeletal muscle physiology. Thus the tissue-specific expression of an AQP does not ensure its physiological significance.

METHODS

Transgenic mice. Transgenic knockout mice deficient in AQP4 protein were generated by targeted gene disruption (18). Measurements were done in litter-matched mice (7–9 wk of age) produced by breeding of AQP4 heterozygotes in a CD1 genetic background. Genotype analysis of tail DNA was done by PCR. Control and “mdx” mice (X-linked muscular dystrophy; C57BL/10ScSn-Dmdmdx/J) were purchased from Jackson Laboratories. The investigators were blinded to genotype information for permeability and functional measurements. All procedures were approved by the University of California, San Francisco Committee on Animal Research.

Water permeability in isolated fiber bundles. Mice were euthanized by an overdose of pentobarbital. The extensor digitorum longus (EDL) (and/or soleus) muscle was immediately removed and incubated for 3–5 min in PBS containing 1 mg/ml collagenase. Small fiber bundles consisting of 2–3 individual muscle fiber segments were microdissected using fine forceps and immobilized on a clean cover glass coated with high-molecular-weight poly-D-lysine (Sigma). The cover glass was mounted in a laminar perfusion chamber in which solution exchange could be accomplished in <1 s. Osmotic water permeability was measured from the time course of cell volume change in response to a change in perfusate osmolality. Fibers were perfused initially with PBS and then with hyposmolar PBS diluted 1:1 with distilled water.

Relative muscle cell volume was determined by a spatial-filtering microscopy method (Ref. 9, see Fig. 3A) based on the sensitivity of a transmitted phase-contrast signal to intracellular refractive index, which depends on cell volume. The perfusate chamber was mounted on the stage of an inverted epifluorescence microscope (Nikon Diaphot) equipped with a phase condenser. The illumination source was a stabilized tungsten-halogen lamp, a 546 ± 10-nm interference filter, and an iris diaphragm to restrict the illuminated region to the fiber. Transmitted light was collected by a ×20 phase objective (Nikon, numerical aperture 0.4), and intensity was measured at 10 Hz by a photodiode (Thor Labs) interfaced to a PC using a 14-bit analog-to-digital converter. The apparatus was vibration isolated and shielded from ambient light to give a stable signal output with <0.1% fluctuations.

Water permeability in fractionated membrane vesicles. Fractionated membrane vesicles were prepared using a modification of previously reported methods (13). Freshly excised skeletal muscle was homogenized in 300 mM sucrose, 5 mM imidazole, and 10 mM Tris·HCl (pH 7.4) containing protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, and 20 μg/ml phenylmethylsulfonyl fluoride) at 4°C using a Polytron homogenizer. After a low-speed centrifugation (1,000 g for 10 min) to remove debris, nuclei, and mitochondria, the supernatant was pelleted (100,000 g for 45 min) and resuspended in 250 mM sucrose and 10 mM Tris·HCl (pH 7.4) containing protease inhibitors by 20 strokes of a glass Dounce homogenizer. The homogenate was centrifuged at 500 g for 10 min at 4°C and adjusted to 1.4 M sucrose, 10 mM Tris·HCl, and 0.2 mM EDTA (pH 7.4). A discontinuous sucrose gradient (2 M sucrose (1 ml), 1.6 M (2 ml), 1.4 M (4 ml, containing homogenate), 1.2 M (4 ml), and 0.8 M (1 ml)) was centrifuged for 2.5 h at 25,000 rpm in an SW27 rotor, and 1 ml fractions were collected. Protein concentration was assayed (Bio-Rad kit) and vesicle size was measured by quasielastic light scattering. AQP4 was assayed in each fraction by immunoblot analysis (see below).

Vesicle osmotic water permeability was measured on an SF-51 stopped-flow apparatus (Wiltshire, UK) by a light-scattering method (27). Suspensions of fractionated vesicles (−0.2 mg protein/ml) in 50 mM sucrose and 5 mM Tris·HCl (pH 7.4) were subjected to a 100 mM inwardly directed sucrose gradient, and the time course of scattered light intensity at 530 nm was measured. The osmotic water permeability coefficient (P) was determined from the light-scattering time course and vesicle diameter as described previously (27).

Immunofluorescence. Mouse EDL skeletal muscle was freshly excised and fixed for 4 h with PBS containing 4% paraformaldehyde, washed extensively in PBS, cryoprotected overnight with PBS containing 30% sucrose, embedded in optimum cutting temperature compound, and frozen in liquid N2. Cryostat sections of 4-μm thickness were mounted on SuperFrost/Plus microscope slides (Fisher). Slides were incubated for 1 h at room temperature with anti-AQP4 antisera (1:100 in PBS containing 1% BSA) that was preabsorbed overnight at 4°C on skeletal muscle sections from AQP4 knockout mice. Rabbit polyclonal antibodies against a COOH terminus peptide were raised as described previously (10). Sections were washed 3 times in PBS and incubated for 45 min with an FITC-conjugated anti-rabbit polyclonal secondary antibody (1:100 dilution; Jackson Immunoresearch). After washing in PBS, slides were incubated in Evans blue (to stain mitochondria) for 30 s before mounting for observation by fluorescence microscopy.

Freeze-fracture electron microscopy. Skeletal muscle tissue samples were fixed overnight in 2% glutaraldehyde in PBS and then washed in PBS. Small pieces of tissue were infiltrated in 30% glycerol for 1 h and frozen in liquid N2-cooled Freon. Muscle samples were fractured in a Balzers freeze-fracture apparatus at −150°C under a vacuum 10−7 Torr and shadowed by platinum at a 45° angle, followed by carbon at 90°. Metal replicas were washed in bleach and water and observed in a Phillips EM 400 electron microscope.

RT-PCR. mRNA was isolated from carefully microdissected mouse EDL and soleus muscle, along with a series of other tissues (kidney, testis, liver, and lung) used as positive controls. After reverse transcription to yield cDNAs, fragments of the coding sequence of each AQP were PCR amplified using gene-specific primers as described previously (20).

Muscle contraction measurements. Electrically stimulated muscle contraction was measured using a force transducer. Freshly isolated EDL muscle bundles were mounted in the measurement apparatus (see Fig. 5A) using 3-0 Nylon suture to secure the ends. The muscle was immersed in oxygenated Krebs solution maintained at 25°C. One end of the muscle was attached to a weight transducer (model TSD125C; Biopac, Santa Barbara, CA) with sensitivity <1 mg, and the other end was secured to the bottom of the container. Container height was adjusted to apply a constant 5-g stretch force to
the unstimulated muscle. The weight transducer was interfaced to a Biopac model MP100A-CE recorder and PC. Data were acquired at a rate of 40 Hz and averaged over 0.2 ms intervals.

A pulse-train generator was constructed for electrical stimulation of muscle contraction. The circuit consisted of two timer chips (LM555; National Semiconductor) connected in tandem (see Fig. 5A). The first timer, wired to operate as an astable multivibrator, produced a train of square pulses. The second timer was wired to operate as a monostable multivibrator. The output of the astable multivibrator was used to trigger the monostable multivibrator. This arrangement made it possible to independently vary pulse frequency and duration between 2–4 Hz and 0.2–2 ms, respectively. Pulse amplitude was specified from the supply voltage (6–15 V). The output was applied to the muscle bundle using thin platinum wire electrodes.

**In vivo treadmill performance.** Skeletal muscle function was evaluated from treadmill performance. Measurements were done on a mouse exercise treadmill system (Harvard Apparatus) equipped with a shock plate at a linear velocity of 29 m/min and a 13° incline (aerobic exercise) and a velocity of 38 m/min and 16° incline (anaerobic exercise). Mice were first trained for two 10-min intervals. Treadmill performance time was defined as the time the mice ran continuously without repeatedly falling back on the shock plate.

**In vivo muscle-swelling assay.** Muscle swelling was studied using an established water intoxication model applied previously in studies of brain edema (14). Mice were injected intraperitoneally with distilled water (20 ml/100 g body wt) containing the V2 agonist 1-desamino-8-D-arginine vasopressin (DDAVP; 0.4 µg/kg) to prevent renal excretion of the water load. This maneuver produces hyponatremia (105–110 mM Na⁺ at 90 min) and consequent osmotically induced organ swelling. After 0 or 90 min, mice were killed, and EDL and liver were excised. Water content was determined by gravimetric measurement of wet-to-dry-weight ratios. After determination of wet weight, samples were dried for 4 h in an oven at 110°C, at which time weight became constant.

**RESULTS**

By indirect immunofluorescence, AQP4 was seen in most skeletal muscle cells from fast-twitch fibers (Fig. 1A) where it was primarily localized to plasma membranes (green staining). Counterstaining with Evans blue, a red fluorescent marker of mitochondria, indicated that the mitochondria-rich slow-twitch fibers (red stained) expressed little or no AQP4. This result is in agreement with previous data in rat (11) that AQP4 is most abundant in fast-twitch skeletal muscle fibers containing relatively few mitochondria, and virtually absent from slow-twitch fibers. As expected, no AQP4 staining was observed in skeletal muscle from AQP4 knockout mice (Fig. 1B). Immunostaining of skeletal muscle from wild-type mice was also done using antibodies against AQP1, AQP2, AQP3, and AQP5. As reported previously (22), AQP1 was expressed in microvascular endothelial cells throughout all sections of skeletal muscle (not shown). No specific staining for AQP2, AQP3, and AQP5 was found in skeletal muscle from wild-type or AQP4 knockout mice, nor did AQP4 deletion affect AQP1 staining. As shown in Fig. 4A, immunoblot analysis confirmed the presence of the ~30 kDa AQP4 protein in skeletal muscle from wild-type mice but not AQP4 knockout mice. To search for small quantities of transcripts encoding each of the mammalian AQPs, RT-PCR was done using mRNAs from mouse EDL and soleus muscle, with positive controls. After reverse transcription to cDNA, PCR amplifications were done with gene-specific primers to amplify

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**Fig. 1.** Immunofluorescence of extensor digitorum longus (EDL) skeletal muscle from wild-type (a) and aquaporin-4 (AQP4) knockout (b) mice, and RT-PCR analysis of AQP transcript expression in skeletal muscle (C). Plasma-lemma localization of AQP4 in wild-type muscle seen as green staining. Strong red staining of mitochondria indicates a slow-twitch muscle fiber that expresses little AQP4. See text for explanations. Scale bar: 50 µm. C: RT-PCR amplification of indicated AQP fragments from EDL (E) and soleus (S) muscle, along with positive controls (C) consisting of a mixture of cDNAs from kidney, testis, liver, and lung.
fragments of the coding sequences of each AQP. Strong bands were seen for AQP1 and AQP4 (Fig. 1C), in agreement with the immunolocalization data. Weak bands were seen for AQP7 and AQP9, which might represent contamination of samples by fat (expressing AQP7) and leukocytes (expressing AQP9). Immunostaining of skeletal muscle for AQP7 and AQP9 proteins is indicated when suitable antibodies are available. Transcripts encoding AQP2, AQP3, AQP5, AQP6, and AQP8 were not detected.

By freeze-fracture electron microscopy, the plasma membrane protoplasmic face of EDL skeletal muscle of wild-type mice showed numerous small OAPs (Fig. 2, arrowheads), in agreement with previous results (28).

OAPs were not detected in skeletal muscle samples from three AQP4 knockout mice.

To determine whether AQP4 deletion resulted in decreased plasmalemma water transport, osmotic water permeability was measured in microdissected EDL fiber bundles as described in methods. Fibers were immobilized on a cover glass and superfused with solutions of different osmolalities (Fig. 3A). The inset shows a photomicrograph of a muscle fiber on the cover glass. Relative cell volume was inferred from transmitted light intensity using spatial-filtering optics. Figure 3B shows representative time course data for muscle cell swelling. For EDL muscle fibers, there was prompt osmotically induced swelling and shrinking with

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Fig. 2. Freeze-fracture electron microscopy of fast-twitch muscle fibers. Orthogonal arrays of particles (arrowheads) were readily identified in skeletal muscle from 3 out of 3 wild-type mice (A), but 0 out of 3 knockout mice (B). Scale bar: 150 nm.

Fig. 3. Osmotic water permeability in microdissected EDL skeletal muscle fiber bundles measured by spatial-filtering microscopy. A: schematic of apparatus for measurement of muscle fiber water permeability. Transmitted light signal provides a real-time semiquantitative measure of muscle cell volume. Inset: micrograph of muscle fiber immobilized on cover glass. B: representative time courses of transmitted light intensity in response to changes in external solution osmolality for EDL or soleus muscle fibers from mice of indicated genotypes. C: half times ($t_{1/2}$, SE, n = 9) for EDL muscle fiber swelling for wild-type mice, AQP4 knockout mice, and mdx mice. Differences not significant.
t_{1/2} = 8 s. Similar results were found for EDL muscle fibers from AQP4 knockout mice and from mdx mice (not shown). The time course of cell swelling and shrinking was slower for soleus muscle fibers from wild-type and AQP4 knockout mice, in qualitative agreement with previous data in rat (11). Measurements done on EDL muscle fibers from a series of wild-type vs. AQP4 knockout mice indicated no effect of AQP4 deletion on muscle cell water permeability (Fig. 3C). The above data suggest that AQP4 expression in skeletal muscle plasmalemma is not associated with increased water permeability. It is not possible to compute absolute plasmalemma osmotic water permeability because of the complex muscle cell ultrastructure with membrane invaginations and intracellular T tubules (2). Further, because of remarkable morphological differences in EDL vs. soleus muscle fibers (6), it is not possible to compare plasmalemma water permeabilities. Apparent swelling rates in EDL fibers may be greater than those for soleus because of the increased plasmalemma surface in EDL.

To avoid these concerns, osmotic water permeability was measured in small, single-walled membrane vesicles isolated from homogenized muscle by differential and sucrose density gradient centrifugation. Immunoblot analysis of vesicle fractions showed strongest AQP4 enrichment in fraction 2, representing plasmalemma vesicles. The fraction 2 vs. homogenate enrichment factor was >5 in three separate sets of studies. AQP4 immunoreactivity was absent in vesicles from AQP4 knockout mice, as expected, and decreased substantially (~10-fold) in vesicles from mdx mice, consistent with previous observations (11, 16). Figure 4B shows stopped-flow light-scattering measurements for two vesicle fractions from wild-type, AQP4 knockout and mdx mice. Apparent t_{1/2} values for the fraction 2 vesicles (~400 ms) were not different in vesicles isolated from EDL of wild-type vs. AQP4 knockout mice. Quasielastic light scattering showed similar unimodal vesicle size distributions of each fraction isolated. For the fraction 2 sample, averaged vesicle diameters were 325 nm (wild type) and 337 nm (knockout), giving P_{1} of 0.0022 cm/s (wild type) and 0.0021 cm/s (knockout). P_{1} was similar (0.0025 cm/s) for vesicles from mdx mice. These P_{1} values are relatively low and typical of membranes not containing water channels. A temperature-dependence study for the fraction 2 vesicles (10–30°C) gave an Arrhenius activation energy of 13.9 kcal/mol, supporting the conclusion that water moves by a lipid-mediated, non-AQP pathway.

The lack of effect of AQP4 deletion/decrease on water permeability can be the consequence of low AQP4 expression in skeletal muscle plasmalemma and/or nonfunctioning of the expressed AQP4. We believe that the former explanation is most likely. Comparative immunoblots of brain vs. skeletal muscle homogenates indicated that skeletal muscle AQP4 protein expression is ~50-fold lower than that in brain (Fig. 4C), consistent with previous observations (11). Also, although difficult to quantify, comparative immunocytochemistry and freeze-fracture electron microscopy indicated that skeletal muscle plasmalemma expresses substantially less AQP4 than brain astroglial plasma membranes and basolateral membranes of kidney-collecting duct.

The experiments above indicate that AQP4 does not contribute significantly to plasma membrane water permeability in skeletal muscle, although they cannot rule out other possible functions of AQP4 in muscle physiology. For this reason, measurements of skeletal muscle function were carried out. In vitro force generation was measured in isolated EDL muscle in response to electrical stimulation by square pulses of 0.2 ms duration, 12 V amplitude, and 40 Hz rate as described in METHODS. Figure 5A shows a schematic of the apparatus and electronic circuit constructed for these measurements. Figure 5B shows representative time courses of force generation in response to intermittent application of pulse trains. A 5-g resting tension was applied in all measurements. Electrical stimulation produced prompt
force generation, with maximal force decreasing upon repetitive stimulation because of progressive ATP depletion. Force generation data were qualitatively similar for EDL of wild-type and AQP4 knockout mice. A series of such data from 10 wild-type and AQP4 knockout mice were analyzed to determine peak force generation, the maximum rate of force generation, and the time for 50% decrease in maximum force during repeated stimulations. For each muscle sample, peak force generation and the maximum rate of force generation were taken as the average of the three largest signal deflections, generally occurring during early stimulations. The rate of force generation was determined from the initial slope of the force curve just after electrical stimulation. The individual and averaged results are summarized in Fig. 6. There was no significant difference in any of these parameters in EDL from wild-type vs. AQP4 knockout mice.

In vivo treadmill performance was measured as an integrated assessment of skeletal muscle function in wild-type vs. AQP4 knockout mice. Performance time was defined as the time the mice remained on the treadmill without repeatedly falling back on a shock plate (see METHODS). Treadmill velocities and incline angles were established to give performance times of 0.5–1 min and ~20 min for wild-type mice, representing an anaerobic and a moderately aerobic exercise stress, respectively. Figure 7 shows no significant differences in treadmill performance times for the wild-type vs. knockout mice. These results are consistent with previous neuromuscular performance studies using an accelerating rotorod (18), and with qualitative observations of mouse spontaneous activity and swimming performance.

As an independent index of integrated muscle physiology, in vivo muscle swelling was measured using an established model of water intoxication (14) in which intraperitoneal water is given with the V2 agonist DDAVP. The percentage increase in muscle water content at 90 min after water infusion was deduced by...
wet-to-dry-weight ratios. Recent results using this water intoxication protocol indicate significant differences in brain swelling in wild-type vs. knockout mice (19), which were attributed to the strong expression of AQP4 in brain at the blood-brain barrier and in astroglial cells. Figure 8 shows the tissue water content before and at 90 min after water infusion for muscle and liver. Liver, which does not express AQP4 protein, was used as a control to normalize for the degree of water intoxication. There was remarkably increased muscle and liver water content at 90 min, but no differences in the increased water content between muscle of wild-type vs. AQP4 knockout mice. Interestingly, tissue water content in wild-type mice was slightly greater than in knockout mice, which may be related to a regulatory effect of AQP4 in the central nervous system. Measurements of serum sodium concentrations indicated comparable degrees of hyponatremia (105–109 mM Na\(^+\)) in the wild-type and AQP4 knockout mice.

### DISCUSSION

The purpose of this study was to test whether AQP4 deletion in mice results in defective skeletal muscle function and decreased plasmalemma water permeability. These experiments were motivated by several observations: the expression of AQP4 in skeletal muscle plasmalemma (10, 11), the decreased OAP density and AQP4 expression in various muscle diseases including hereditary muscular dystrophies (21, 29, 30), and the greater AQP4 expression and apparent water permeability in fast- vs. slow-twitch muscle fibers (11). In addition, physiological measurements showing substantial muscle swelling and intracellular osmolyte production during exercise (5, 12, 15) suggested a possible physiological need for rapid water movement across myocyte plasma membranes. Our study utilized AQP4 knockout mice, which have normal development, growth, and appearance compared with wild-type mice (18). Phenotype analysis of AQP4 knockout mice indicated a mild defect in urinary concentrating ability (18) due to fourfold reduced transepithelial osmotic water permeability in inner medullary collecting duct (4). Recent experiments showed that AQP4 deletion in brain resulted in deceased brain swelling after acute water intoxication and ischemic stroke (19) and that AQP4 deletion in lung produced a small decrease in airspace-capillary water permeability (25). The comparison of skeletal muscle phenotype in wild-type vs. AQP4 knockout mice permitted direct analysis of the role of AQP4 in skeletal muscle function.

Data from several in vitro and in vivo experimental approaches suggested that AQP4 does not play a significant role in skeletal muscle physiology and pathophysiology. Skeletal muscle plasmalemma water permeability was measured from osmotically induced volume changes in intact muscle fibers and fractionated membrane vesicles. AQP4 deletion did not affect water permeability, probably because of the very low expression of AQP4 in skeletal muscle compared with brain and inner medullary collecting duct in kidney. AQP4 deletion did not affect skeletal muscle contractile function in measurements of electrically stimulated muscle contraction and in vivo treadmill performance. In addition, wild-type and AQP4 knockout mice were indistinguishable in an in vivo water intoxication model of muscle swelling. Finally, water permeability was not different in skeletal muscle from wild-type mice vs. mdx mice, a dystrophin-deficient mouse model of muscular dystrophy with decreased AQP4 protein expression and OAP density.

In summary, the results here provide evidence against a role for AQP4 in skeletal muscle function and in the pathophysiology of hereditary and acquired muscle disease. AQP4 expression in skeletal muscle may represent a vestigial remnant from an ancient time when high muscle water permeability was required. The decreased AQP4 expression in diseased muscle cells is probably an epiphenomenon rather than a causal factor involved in disease pathogenesis or pathophysiology. Although we believe it unlikely, the possibility cannot be ruled out that AQP4 deletion might produce defective skeletal muscle function with physiological stresses not tested here, or that AQP4 may be important in human skeletal muscle physiology but not in mice. Nor can the possibility be ruled out that AQP4 might serve a non-water-transporting role in skeletal muscle, or in membrane structure/organization.

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Address for reprint requests and other correspondence: A. S. Verkman, 1246 Health Sciences East Tower, Cardiovascular Research Institute, Univ. of California, San Francisco, San Francisco, CA 94143-0521 (E-mail: verkman@itsa.ucsf.edu; web address: http://www.ucsf.edu/verklab).
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