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Expression and Characterization of Lacrimal Gland Water Channels in *Xenopus* Oocytes

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Lacrimal grands transport fluid for secretion as tears. To examine whether the expression of the aquaporin family of water channels is essential for water transport in lacrimal glands, we determined the water permeability in *Xenopus* oocytes injected with rat lacrimal gland poly(A)$^+$ RNA. In oocytes injected with poly(A)$^+$ RNA, osmotic water permeability was 4-fold higher than that observed in vehicle-injected controls. The enhanced water permeability was inhibited by 65% by coinjection of poly(A)$^+$ RNA with antisense aquaporin-5 but not antisense aquaporin-1 oligonucleotide. To detect aquaporin mRNA in rat lacrimal glands, we performed reverse transcription-polymerase chain reaction analysis. PCR products were detected using specific aquaporin-5 primers. Our results strongly suggest that rat lacrimal glands express aquaporin-5 water channels for lacrimation.

Changes in the rate of transport of water into and out of cells are involved in many physiological events. The driving force for water movement is thought to be provided by osmotic gradients, i.e. simple diffusion through the lipid bilayer (1). However, the molecular pathways for water transport have not yet been completely characterized. Recently, five different cDNAs encoding functional water-selective molecular channels have been identified in mammals, and this group of proteins has been referred to as the ''aquaporins''(2–3). These proteins were expressed abundantly in secretory tissues and glands, suggesting that the water channel family is essential for water transport in several tissues. Aquaporins were also found in eye components including the ciliary epithelium, lens epithelium, cornea epithelium and endothelium, trabecular meshwork, canal of Schlemm and lacrimal gland(4). Therefore, aquaporins may play key roles in the maintenance of fluid balance in the ocular tissues. Here, we report on functional evidence for expression of water channels in the lacrimal gland. *Xenopus* oocytes injected with rat lacrimal gland poly(A)$^+$RNA showed an increase in water permeability that was abolished by an aquaporin-5 antisense oligonucleotide.

MATERIALS AND METHODS

Preparation of lacrimal gland mRNA. Exorbital lacrimal glands were isolated from male SD rats. Total RNA was isolated using a Pharmacia RNA purification Kit. Poly(A)$^+$RNA was purified by affinity chromatography on oligo(dT)-cellulose as described previously(6).

Preparation of oocytes. Oocytes were prepared as described by Taylor et al.(7). Mature female *Xenopus laevis* were anesthetized, oocytes(Stage V-VI) were removed and placed in Ca^{2+}-free Barth's buffer. The oocytes were then defolliculated by gentle agitation for 1 hr in Ca^{2+}-free Barth's buffer containing 2 mg/ml of type II collagenase. After the oocytes were washed sufficiently with Barth's buffer, they were completely denuded. The oocytes were incubated overnight at 18°C in Barth's buffer and injected with RNA or water the following day.

Microinjection of mRNA in oocytes. Fifty nl of water or mRNA (1 mg/ml) in water was microinjected into oocytes using a Drummond microinjection system with sterile glass micropipettes. Oocytes were maintained at 18°C in Barth's

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Abbreviations used: Pf, osmotic water permeability.

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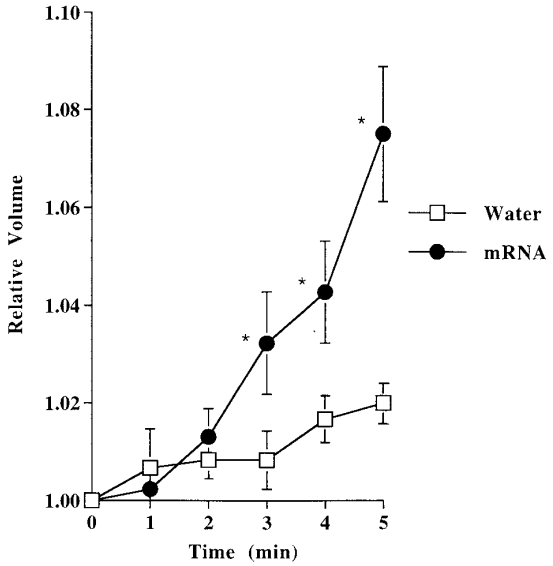

FIG. 1. Time course of osmotic swelling of individual oocytes. Three days after microinjection of 50 nl of water (□) or rat lacrimal gland mRNA (●), the oocyte volume was calculated as described in Materials and Methods. Values represent means ± S.E.M. *$P < 0.01$ vs water.

buffer with daily buffer changes. In some experiments, mRNA was coinjected with antisense aquaporin oligonucleotide which was designed as follows: aquaporin-1 (5′-CCAGAAGAGCTTCTTCTTGAACTCGCTGGC-3′) corresponding to nucleotides +4 ∼ +34 (8) and aquaporin-5 (5′-GAAGAAGGCAAGGGAGCACACCTCCTT-3′) corresponding to nucleotides +4 ∼ +31(5).

Oocyte volume and osmotic water permeability. Three days after injection, oocytes were transferred from 200 mOsm to 20 mOsm modified Barth's buffer, and osmotic volume change was observed at 18 °C by transmitted light under an Olympus phase-contrast microscope. Oocyte views were recorded at 1-min intervals, and oocyte volume was calculated from the images on a C-IMAGING 1280 image-processing system. Osmotic water permeability (Pf) was determined from the initial slope of the time course of V/V_0 ($d(V/V_0)dt$), the initial oocyte volume ($V_0 = 9 \times 10^{-4}$ cm^3), the initial oocyte surface area ($S = 0.045$ cm^2) and the molar volume of water ($V_w = 18$ cm^3/mol): $Pf = [V_0 \times d(V/V_0)dt]/[S \times V_w \times (mOsm_{in} - mOsm_{out})]$.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Rat lacrimal gland poly(A)$^+$RNA was reverse transcribed using oligo $d(T)_{18}$ primer, and the first-strand cDNA preparations were used as templates for PCR. Specific oligonucleotide DNA primers were based on the rat aquaporin cDNA sequences (9-11): sense primers, aquaporin-1 (5′-CCTGAATTCATGGCCAGCGAGTTCAAG-3′), aquaporin-2 (5′-GGAATTCATGTGGGAACTCAGA-3′), aquaporin-3 (5′-CGAATTCATGCTCCACATCCGCTA-3′), aquaporin-4 (5′-GGAATTCATGAGTGACGGAGCTG-3′) and aquaporin-5 (5′-CAAGAATTCATGAAAAAGGAGGTGTGC-3′); reverse primers, aquaporin-1 (5′-GCCGGATCCCTTCTATTTGGGCTTCAT-3′), aquaporin-2 (5′-GGGATCCTCAGGCCTTGCTGCC-3′), aquaporin-3 (5′-TGGATCCTCAGATCTGCTCCTTGT-3′), aquaporin-4 (5′-GTCTAGATCTTACAGAAGATAATA-3′) and aquaporin-5 (5′-GGATCCAATGCCTCTTCCCCAGCT-3′). Glyceraldehyde-3-phosphodehydrogenase (G3PDH) primers were designed as follows: sense primer (5′-ACCACAGTCCATGCCATCAC-3′) and reverse primer (5-TCCACCACCCTGTTGCTGTA-3′). The reverse transcribed RNA was amplified by 30 cycles of polymerase chain reaction (1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C) using each primer (50 pmol) and Taq polymerase (Pharmacia). The amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

Following injection of lacrimal gland mRNA, *Xenopus* oocytes will express certain proteins and exhibit increased water permeability if aquaporins are expressed in the lacrimal gland. Figure 1 shows the time course of oocyte swelling in response to transfer of oocytes from Barth's buffer (200mOsm) to a 10-fold dilution of Barth's buffer (20mOsm) with distilled water at 18°C. Oocyte volume was determined in 1-min intervals by image analysis. Under

TABLE 1
Inhibition of Osmotic Water Permeability (Pf) by Antisense Aquaporin Oligonucleotides in Oocytes Injected with Lacrimal Gland mRNA

Injection	Pf ($\times 10^{-4}$ cm/s)
Water	11.5 ± 4.4
mRNA	51.2 ± 9.5**
mRNA + aquaporin-1 antisense	49.5 ± 9.4**
mRNA + aquaporin-5 antisense	23.0 ± 4.8##
Aquaporin-5 antisense	17.1 ± 6.8

Values represent means ± SEM.
** $P > 0.01$ vs. water.
$P > 0.01$ vs. mRNA.

these conditions, vehicle(H_2O)-injected oocytes showed a slight increase in volume about five minites after hypotonic stimulation. In contrast, the osmotic water permeability (Pf) of mRNA-injected oocytes was significantly (4-fold) higher than that observed in vehicle-injected oocytes. These results suggest the presence of message(s) encoding water transporter-like protein(s) in lacrimal glands.

We then examined whether increased water permeability in *Xenopus* oocyte was induced by translation of aquaporin mRNA. Lacrimal gland mRNA was coinjected with an antisense aquaporin oligonucleotides. The increase in oocyte Pf due to injection of lacrimal gland mRNA was blocked by 65 % with coinjection of antisense aquaporin-5 oligonucleotide (10 ng) but not antisense aquaporin-1 oligonucleotide (Table 1). These results strongly suggest that the increase in oocyte water permeability is mediated by aquaporin-5. To confirm the expression of aquaporin in lacrimal grands, the detection of aquaporin mRNA was performed by RT-PCR. PCR was also performed using primers for G3PDH as a control. The results are shown in Figure 2. Amplified band were detected using primers for specific aquaporin-5 and G3PDH but not these for aquaporin-1, -2, -3 or -4. This finding suggests that the water channels expressed in lacrimal glands are composed of only aquaporin-5 and/or its homolog(s), in agreement with the observation that the increase in water permeability in oocytes was abolished by an antisense aquaporin-5 oligonucleotide.

In the present study, the water permeability of oocytes injected with lacrimal gland mRNA increased, and this increase in water permeability was abolished by antisense aquaporin-5 oligonucleotide. Recently, Kuwahara et al.(12) reported the regulatory mechanism of aquaporin-2, the vasopressin-regulated water channel in the kidney collecting ducts. Aquaporin-2 protein is phosphorylated by a cAMP-dependent protein kinase, which results in an increase

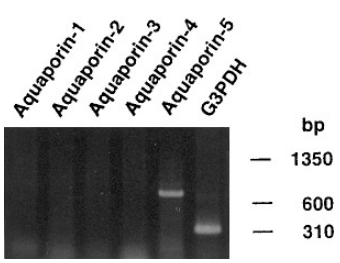

FIG. 2. Expression of aquaporin-1, -2, -3, -4, -5 and glyceraldehyde-3-phosphodehydrogenase (G3PDH) mRNA in rat lacrimal glands. RT-PCR products were electrophoresed on a 1.5 % agarose gel and stained with ethidium bromide.

in water permeability through the apical membrane for urine concentration. Phosphorylation sites for cAMP-dependent protein kinase and protein kinase C are present in aquaporin-5 (5). Furthermore, lacrimation is stimulated by the autonomic nervous system and several hormones, which act via second mesengers such as cAMP (1). Thus, the existence of a consensus phosphorylation sites in aquaporin-5 suggests that it may also be under similar regulatory controls, contributing to the secretion of tears. Further studies are necessary to determine the mechanism of regulation of aquaporin-5 water channel, whether phosphorylation by the protein kinase is involved in the regulation of aquaporin-5, and whether autonomic neuronal stimulation affects the function of aquaporin-5.

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