Effects of acetazolamide and anordiol on osmotic water permeability in AQP1-cRNA injected *Xenopus* oocyte[1]

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ABSTRACT

AIM: To study the effects of acetazolamide and anordiol on osmotic water permeability in aquaporin 1 (AQP1)-cRNA injected *Xenopus* oocyte and their mechanisms. **METHODS:** AQP1 gene constructed in pBluescript was transcripted into cRNA *in vitro* and then the cRNA was injected in *Xenopus* oocytes. The effects of acetazolamide and anordiol on the water transport function of AQP1 were observed by assaying the osmotic swelling of oocytes. In addition, their effects on protein expression of AQP1 were quantitatively investigated by Western blotting method. **RESULTS:** After incubation for 15 min or 72 h, acetazolamide, a carbonic anhydrase inhibitor, equally reduced the water permeability of AQP1-cRNA injected oocyte in a dose-dependent manner. After incubation for 72 h, anordiol, an antiestrogen with partial estrogenic activity, reduced the osmotic water permeability dose dependently as well; however, no discernable action was observed after incubation with anordiol for 15 min. The Western blotting analysis showed that acetazolamide did not influence the protein expression of AQP1. However, after incubation for 72 h with anordiol (10 μmol/L), the quantity of AQP1 in the oocyte membrane was decreased dramatically ($P<0.05$). **CONCLUSION:** Both acetazolamide and anordiol inhibited the osmotic water permeability of AQP1-cRNA injected oocyte, but their mechanisms were different. Acetazolamide functionally inhibited the osmotic water permeability of AQP1, whereas anordiol primarily decreased the amount of AQP1 protein in the oocyte membrane.

INTRODUCTION

So far, at least 10 aquaporins (AQP) numbered 0 through 9, have been identified from various mammalian tissues[1]. They play a critical role in the regulation of water transport across cellular membranes and belong to the major intrinsic protein (MIP) family that forms a pore highly selective for water transport. It is becoming apparent that aquaporin biology will be related to the pathophysiology and perhaps even therapy of a wide array of conditions[2]. Aquaporin 1 (AQP1) is the first characterized water channel molecule, which was serendipitously discovered during studies of the human red cell Rh protein by Agre and his colleagues[3]. AQP1 is distributed in variant tissues including the renal proximal convoluted tubule epithelia, red blood cell, the apical membrane of the brain choroid plexus epithelia, ciliary body epithelia, cornea endothelia, hepatobiliary epithelia, sweat gland and salivary gland epithelia, bronchus and alveolus epithelia, colon, spleen, pancreas and capillary endothelia[4-7], indicating its im-

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portant role in the formation of urine, generation of aqueous humor, cerebrospinal fluid balance and the fluid secretion and absorption in other tissues. There are evidences showing that AQP1 plays an important role in several water balance disorder diseases[2], which suggests AQP1 might be a new therapeutic target for human diseases in the future. For example, AQP1 inhibitors especially in combination with conventional salt transport-blocking diuretics might be highly effective in inducing a diuresis in refractory states of edema associated with congestive heart failure and cirrhosis. Furthermore, AQP1 inhibitors are predicted to correct the renal proximal tubule hyper-reabsorption in these conditions as well as to interfere with the counter-current exchange mechanism that generates the hypertonic medullary interstitium[8].

The earliest blockers of water permeability through water channel aquaporins are mercurial reagents such as $HgCl_2$. For AQP1, inhibition by mercury has been attributed to the formation of a mercaptide bond with cysteine residue 189 found in the putative pore-forming region loop E[9]. Interestingly, after reviewing the published papers, we found that the tissue distribution and even the subcellular localization of AQP1 are similar to that of some carbonic anhydrase isoenzymes, suggesting the potential relationship between the two kinds of proteins in their structures or functions. Our previous studies showed that acetazolamide, a kind of sulfanilamide used as a carbonic anhydrase inhibitor, could inhibit the gene expression of AQP1 in rat kidney. In addition, we have found that anordiol, an antiestrogen with partial agonist activity, could regulate the gene expression of AQP1 in rat uterus, which might be a mechanism of water imbibition of uterine cell[10].

In present study, we aimed to study the effects of acetazolamide and anordiol on osmotic water permeability in AQP1-cRNA injected *Xenopus* oocyte and their mechanisms, and to discover more AQP regulators.

MATERIALS AND METHODS

Animals Adult female *Xenopus laevis* weighing 150±10 g (obtained from Institute of Developmental Biology, Chinese Academy of Sciences) were kept in plexiglas tanks containing carbon-filtered water and were fed frog chow twice a week.

Chemicals and solutions Anordiol (AF-45, 2α, 17α-diethynyl-A-nor-5α-androstane-2β,17-diol) was obtained from the Shanghai Institute of Materia Medica, China, and was dissolved in Modified Barths' Saline (MBS). Acetazolamide (Sigma, St Louis, MO) was dissolved in MBS. Tricaine and collagenase (type IA) were obtained from Sigma, St Louis, MO. MBS: NaCl 88 mmol/L, KCl 1 mmol/L, $NaHCO_3$ 2.4 mmol/L, HEPES-NaOH 15 mmol/L, pH 7.6, $Ca(NO_3)_2$ 0.3 mmol/L, $CaCl_2$ 0.41 mmol/L, $MgSO_4$ 0.82 mmol/L, sodium penicillin 10 mg/L, streptomycin sulfate 10 mg/L, gentamycin sulfate 100 mg/L, and nystatin 10 U/mL. Transfer buffer: Tris-HCl 25 mmol/L, glycine 192 mmol/L, and methanol 20 % pH 8.3. TBS: Tris-HCl 100 mmol/L, NaCl 0.9 %, pH 7.5. Hypotonic lysis buffer: Na_2HPO_4 7.5 mmol/L, pH 7.4, edetic acid 1 mmol/L buffer containing phenylmethylsulfonyl fluoride 20 mg/L, pepstatin A 1 mg/L, leupeptin 1 mg/L, diisopropylfluorophosphate 1:2000.

Isolation of oocytes *Xenopus* oocytes in stage V-VI were harvested according to established procedures[11] except minor changes. Briefly, the frog was anesthetized by immersing in water containing 0.2 % tricaine (3-aminobenzonic acid ethyl ester) for 5 min, followed by hypothermia induced by placing the frog over ice. A 1-cm incision was made in the abdominal wall, and a lobe of ovary was excised. The excised piece of ovary containing oocytes was rinsed several times with Ca^{2+}-free MBS until the solution was clear. The tissue was then agitated in about 15 mL sterile filtered Ca^{2+}-free MBS containing collagenase (type IA, 2 g/L) for 30-40 min. Free oocytes were rinsed several times with sterile Ca^{2+}-free MBS, sorted, and then stored in MBS at 18 ºC.

Preparation of cRNA The cDNA encoding AQP1 was a generous gift from Dr Fischbarg J (Columbia University, USA). It had been cloned into the expression plasmid pBluescript. Capped cRNA was synthesized using a T3 RNA polymerase kit (Promega) after a digestion with KpnI to linearize the plasmid, and the cRNA was purified by phenol-chloroform extraction. The cRNA concentration was determined by ultraviolet absorbance, and its quality was assessed by gel electrophoresis[12].

Microinjection of cRNA into oocytes and measurement of Pf One day after isolation, oocytes were injected with either 50 nL of water (sham injection) or 50 nL of water containing 10 ng of AQP1-cRNA (as control). After injection, oocytes were kept for 72 h at 18 ºC in MBS. To determine the proper time when the drugs exert their best effects, acetazolamide (final concentrations: 0.1, 1, or 10 μmol/L) or anordiol (final

concentrations: 0.1, 1, or 10 μmol/L) was added to MBS immediately after the injection of AQP1-cRNA, and then incubated with oocytes for 72 h; for other groups acetazolamide (final concentrations: 0.1, 1, or 10 μmol/L) or anordiol (final concentrations: 0.1, 1, or 10 μmol/L) was added to MBS 72 h after the injection of AQP1-cRNA, and then incubated with oocytes for 15 min. $HgCl_2$ was used as a positive control reagent.

Osmotic swelling was performed at 22 ºC following transfer of the oocytes from 200 mOsM (OsM_{in}) to 70 mOsM (OsM_{out}) MBS diluted with deionized water. Sequential oocyte images were photoed at 30 s intervals for a total of 3 min or until just before the oocyte membrane ruptured, and the volumes of the sequential images were calculated on an 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 ($V_0=9\times10^{-4}$ cm^3), the initial oocyte surface area (S=0.045 cm^2) and the molar volume of water (Vw=18 cm^3/mol) [13]: $Pf=[V_0\times d(V/V_0)/dt]/[S\times V\text{w}\times(OsM_{in}-OsM_{out})]$.

Oocyte membrane isolation and Western blotting analysis The membranes of oocytes were isolated according to established procedures[14]. Briefly, groups of 20 oocytes were transferred with MBS into 1.5 mL microcentrifuge tubes on ice. After chilling for ≥5min, the buffer was removed and the oocytes were lysed in 1 mL of ice-cold hypotonic lysis buffer by repeatedly vortexing and pipetting the samples. The yolk and cellular debris were pelleted at 750×*g* at 4 ºC for 5 min. The membranes were then pelleted from the supernatant at 16 000×*g* at 4 ºC for 30 min. The floating yolk was removed from the top of the tubes with a cotton applicator, and the supernatant was removed. The membrane pellets were gently washed once with an equal volume of ice-cold hypotonic lysis buffer and were resuspended in 10 μL 1.25 % (*w/v*) SDS/oocyte. Then samples were solubilized in sample buffer and heated to 60 ºC for 15 min. Total protein concentrations were measured by Lowry method using bovine serum albumin as the standard. Each sample containing 30 μg protein was separated by 12 % SDS-polyacrylamide gel. Proteins were transferred electrophoretically from gels to nitrocellulose membranes performed in a transfer buffer, using a Bio-Rad Mini-Trans-Blot cell. The blots were blocked for 30 min with 5 % nonfat dry milk in TBS and incubated overnight at 4 ºC with an affinity-purified anti-AQP1 polyclonal antibody (a gift from Dr He-Ming YU) in TBS containing 2.5 % nonfat dry milk. The membranes were washed three times with TBS containing 0.1 % Tween-20 (TBST) and incubated for 2 h with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase 1:5000 diluted in TBS containing 2.5 % nonfat milk. After washing three times for 5 min with TBST, AQP1 was stained with BCIP/NBT. Stained bands were scanned and pixel intensity was quantified using Gel Doc 2000 Image system.

Statistical analysis Data were presented as mean±SD. Significance of differences was determined by one-way analysis of variance (ANOVA), followed by *t*-test. $P<0.05$ was considered to be statistically significant.

RESULTS

Effect of acetazolamide on osmotic water permeability of AQP1 As shown in Fig 1 and Fig 2, AQP1-cRNA injected (control) oocytes demonstrated rapid osmotically driven increase in relative volumes. In contrast, oocytes incubated with 1 μmol/L and 10 μmol/L acetazolamide for 15 min or 72 h showed marked reduction of relative volume increase after being transferred into 70 mOsM MBS, suggesting that acetazolamide could inhibit the osmotically induced volume swelling. The osmotic water permeability inhibitory effects of identical concentration of acetazolamide at the two time points were almost the same. After incubation of the oocytes for 15 min with acetazolamide (10 μmol/L), the reduction of relative volumes was similar to that obtained from 15 min of incubation in $HgCl_2$ (0.3 mmol/L). Sham-injected oocytes did not show any appreciable increase in relative volumes.

Increased concentrations of acetazolamide showed a dose-dependent inhibitory effect on the osmotic water permeability of AQP1-cRNA injected oocytes at the both time points. At the highest concentration tested, 10 μmol/L acetazolamide (15 min) reduced water permeability of AQP1 by an average value of 81% [Pf 10 μmol/L acetazolamide=$(35\pm9)\times10^{-4}$ cm/s; cf. Pf control=$(188\pm15)\times10^{-4}$ cm/s], similar to the effect of 0.3 mmol/L $HgCl_2$ (Fig 3).

Effect of acetazolamide on protein expression of AQP1 The protein expression of AQP1 in oocyte membrane was analyzed by Western blotting. Membrane fractions prepared from AQP1-cRNA injected oocytes with different treatments all contained immunoreactive protein bands, showing that the protein was

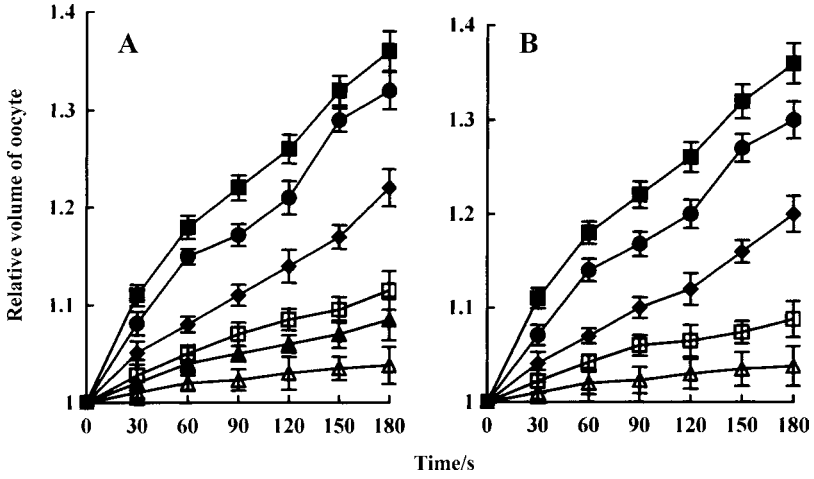

Fig 1. Acetazolamide block of osmotically induced swelling in oocytes expressing AQP1. Relative volumes were determined using videomicroscopy as described in methods after the transfer of oocytes from MBS (200 mOsM) into hypotonic MBS (70 mOsM) at time 0. AQP1-cRNA injected oocytes were incubated for 15 min (A) or 72 h (B) in MBS (■) (as control), MBS with different concentration of acetazolamide: 0.1 μmol/L (●), 1 μmol/L (◆), 10 μmol/L (□) or MBS with 0.3 mmol/L $HgCl_2$ (▲). Sham-injected (△) oocytes were incubated in MBS. *n*=15. Mean±SD.

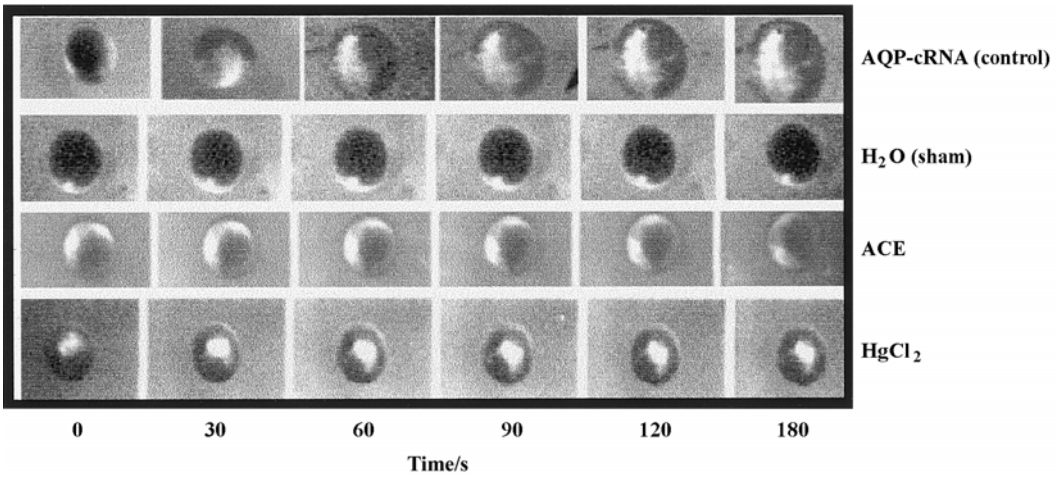

Fig 2. Regulation of acetazolamide on water transportation in AQP1-cRNA injected oocytes. Photographing began after the transferring of oocytes from MBS (200 mOsM) into hypotonic MBS (70 mOsM) at time 0. AQP1-cRNA injected oocytes were incubated for 15 min in MBS with acetazolamide (ACE 10 μmol/L) or $HgCl_2$ (0.3 mmol/L). Sham-injected oocytes were incubated in MBS.

expressed in the membrane. The 28 kDa (AQP1) band of Western blotting was quantified by densitometry. The results showed that acetazolamide at the dose of 0.1-10 μmol/L did not inhibit the protein expression of AQP1 in oocyte. The quantities of AQP1 expressed in AQP1-cRNA injected oocytes incubated with acetazolamide both for 72 h and 15 min were similar to that in control (Fig 4).

Effect of anordiol on osmotic water permeability of AQP1 Oocytes in control showed rapid osmotically driven increase in relative volumes. Oocytes incubated with 10 μmol/L anordiol for 72 h showed marked reduction of relative volume increase after being transferred into 70 mOsM MBS, and the level of reduction was higher than that produced by incubating for 15 min with 0.3 mmol/L $HgCl_2$. But oocytes that were incubated with anordiol (0.1, 1, or 10 μmol/L) for 15 min did not show any reduction in the rate of relative volume increases. These results suggested that anordiol did not affect the osmotic permeability of AQP1 that has been expressed in the membrane of oocytes. Sham-injected oocytes did not show any appreciable

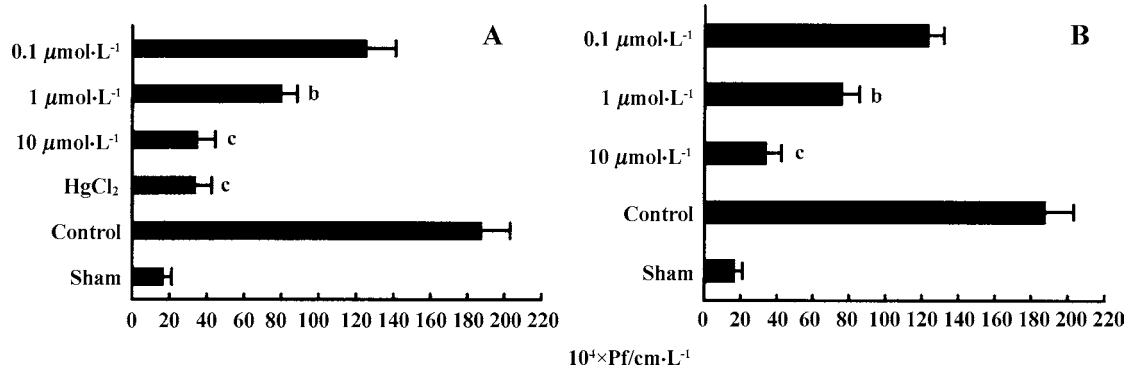

Fig 3. Dose-response relationship for the effect of acetazolamide on Pfs of oocytes expressing AQP1. cRNA injected oocytes were incubated for 15 min (A) or 72 h (B) in MBS containing acetazolamide at the indicated concentrations. Volumes were monitored every 30 s after the transfer of each oocyte into hypotonic MBS (70 mOsM) containing the same respective concentration of acetazolamide. *n*=15. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

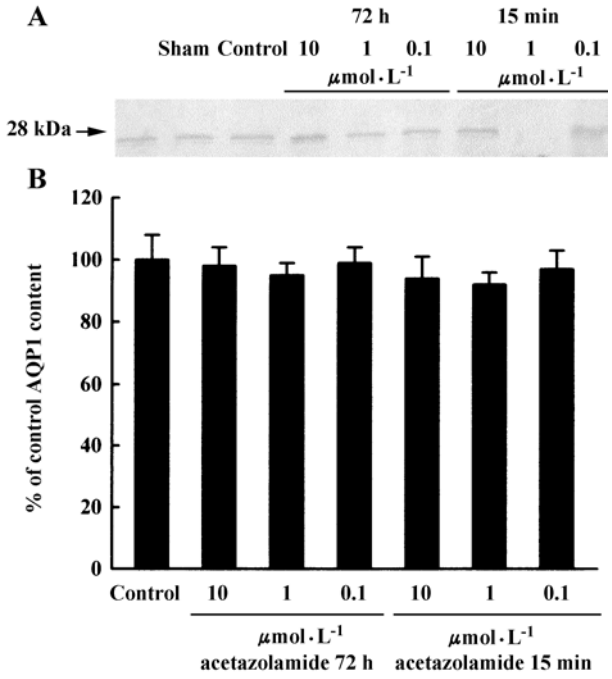

Fig 4. Western blotting analyses of AQP1 expression in plasma membranes of oocytes injected with AQP1-cRNA after treatment with acetazolamide. Each lane was loaded with 30 μg of total membrane protein prepared from 20 oocytes. A: a representative figure of Western blotting. The position of the 28 kD molecular mass marker is indicated on the left. B: The 28 kDa band of Western blotting was analyzed by densitometry, and the values were expressed as percent content of AQP1 in pretreated oocytes relative to that of control oocytes. *n*=6. Mean±SD.

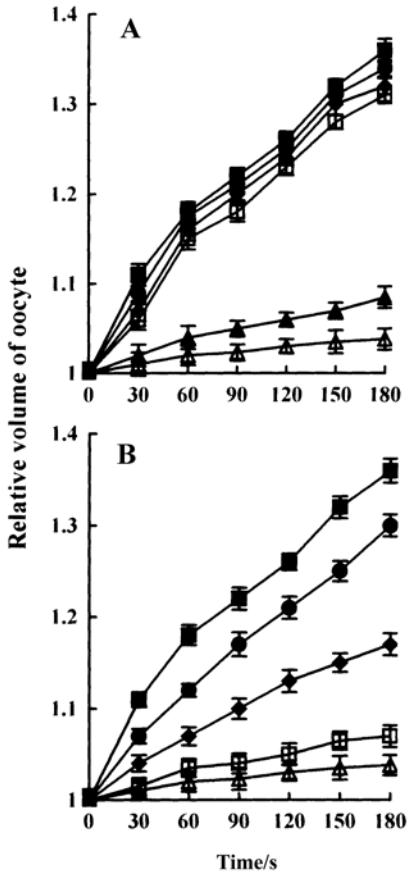

Fig 5. Effect of anordiol on osmotically induced swelling in oocytes expressing AQP1. Relative volumes were determined using videomicroscopy as described in methods after the transfer of oocytes from MBS (200 mOsM) into hypotonic MBS (70 mOsM) at time 0. AQP1-cRNA injected oocytes were incubated for 15 min (A) or 72 h (B) in MBS (■) (as control), MBS with different concentration of anordiol: 0.1 μmol/L (●), 1 μmol/L (◆), 10 μmol/L (□) or MBS with 0.3 mmol/L $HgCl_2$ (▲). Sham-injected (△) oocytes were incubated in MBS. *n*=15. Mean±SD.

increase in relative volumes (Fig 5).

After incubation for 72 h, increased concentrations of anordiol showed a dose-dependent inhibitory effect on the osmotic water permeability of AQP1-cRNA injected oocytes. Oocytes incubated with 10 μmol/L

anordiol for 72 h yielded Pf lower than that of oocytes incubated with 0.3 mmol/L $HgCl_2$ for 15 min [Pf 10 μmol/L anordiol=$(25\pm5)\times10^{-4}$ cm/s; cf. Pf 0.3 mmol/L $HgCl_2$= $(34\pm8)\times10^{-4}$ cm/s]. However, oocytes incubated with all three doses of anordiol for 15 min yielded Pfs that were similar to that of oocytes in control (Fig 6).

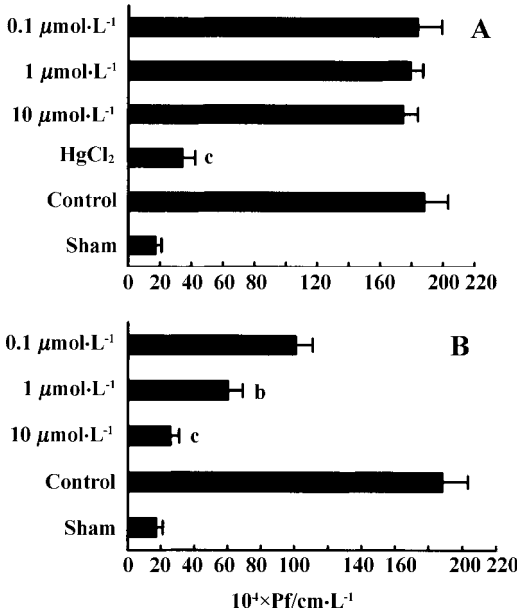

Fig 6. Dose-response relationship for the effect of anordiol on Pfs of oocytes expressing AQP1. cRNA injected oocytes were incubated for 15 min (A) or 72 h (B) in MBS containing anordiol at the indicated concentrations. Volumes were monitored every 30 s after the transfer of each oocyte into hypotonic MBS (70 mOsM) containing the same respective concentration of anordiol. *n*=15. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control.

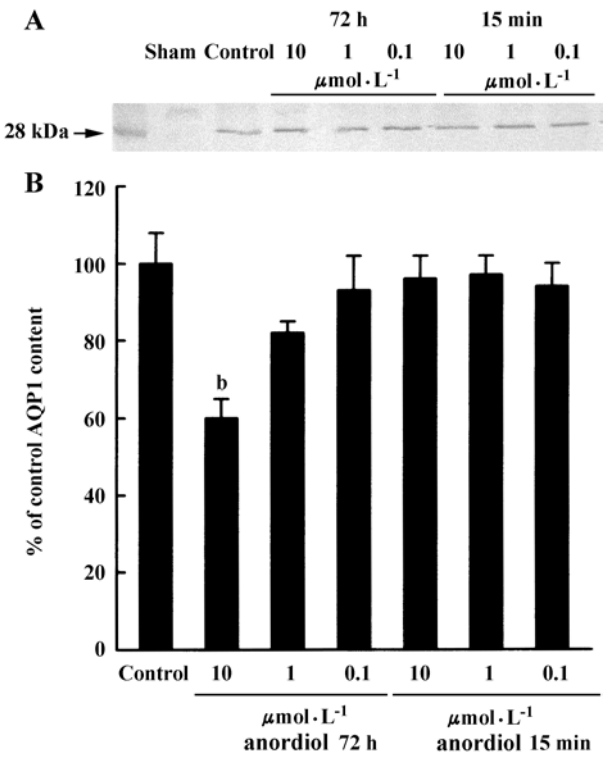

Fig 7. Western blotting analyses of AQP1 expression in plasma membranes of oocytes injected with AQP1-cRNA after treatment with anordiol. Each lane was loaded with 30 μg of total membrane protein prepared from 20 oocytes. A: a representative figure of Western blotting. The position of the 28 kDa molecular mass marker is indicated on the left. B: The 28 kDa band of Western blotting were analyzed by densitometry, and the values are expressed as percent content of AQP1 in pretreated oocytes relative to that of control oocytes. *n*=6. Mean±SD. $^bP<0.05$ *vs* control.

Effect of anordiol on protein expression of AQP1 The 28 kDa (AQP1) band of Western blotting was quantified by densitometry. The quantities of AQP1 expressed in oocytes treated with anordiol for 72 h were decreased, and increased concentrations of anordiol showed a dose-dependent expression inhibitory effect. But treatment with anordiol (all tested doses) for 15 min did not affect the quantities of AQP1 expressed in oocyte membrane (Fig 7).

DISCUSSION

The lipid membranes of native *Xenopus* oocytes have a very low permeability to water, since there is no AQP on it[15]. Therefore, when the AQP1-cRNA injected into the oocytes and successfully translated in AQP1 water channel, the oocytes could swell in volume or rupture after being transferred to hypotonic solution. In this study, AQP1 capped cRNA was synthesized *in vitro* and injected into oocytes. 72 h later, when transferred from a 200 mOsM to a 70 mOsM solution, oocytes injected with AQP1-cRNA swelled significantly and ruptured within 5 min, but water-injected oocytes swelled minimally and failed to rupture even after incubations for >30 min. The Western blotting analysis also demonstrated that injecting cRNA for AQP1 has been proven to be very effective for expressing this water channel. $HgCl_2$ has been known as an inhibitor of water channel. In the analysis of osmotic swelling, we found that $HgCl_2$ significantly decreased the osmotic permeability of AQP1. All of these results indicated that we successfully established an AQP protein translating system using *Xenopus* oocytes.

Acetazolamide is a carbonic anhydrase (CA) inhibitor, and it is the only diuretic that acts on the renal proximal tubules. The diuretic activity of acetazolamide is due to its potent inhibition of both CAII and CAIV, resulting in nearly complete abolition of $NaHCO_3$ reabsorption in the proximal tubule. However, the weak action and the rapid development of tolerance have limited its use as a diuretic. At present, acetazolamide is mainly used for edematous diseases such as glaucoma, mountain sickness, congestive heart failure induced or drug-induced edema[16]. Acetazolamide has been used orally for the reduction of intraocular pressure (IOP) in patients suffering from glaucoma for many years. It is used to relieve the acute symptoms of open-angle glaucoma, prolong the onset of blindness in persons with advanced glaucoma and reduce IOP preoperatively by reducing the aqueous humour formation[17]. AQP1 is abundant in both the anterior ciliary epithelium[4] and canals of Schlemm[4,18], which suggests a role in secretion and uptake of aqueous humour. The investigations of our laboratory have indicated that acetazolamide inhibited gene expression of AQP1 in rat kidney[19,20]. In this study, we discovered that acetazolamide could inhibit the osmotic water permeability of AQP1-cRNA injected oocytes in a dose-dependent manner. But acetazolamide did not change the quantity of AQP1 expressed in the oocyte membrane. These results suggested that the inhibitory effect of acetazolamide on water permeability might not be attributed to reducing AQP1 protein expression, but to inhibit the water transportation of AQP1 in AQP1-cRNA injected oocyte. Recent studies provided an evidence for the involvement of AQP in IOP regulation by facilitating aqueous fluid secretion across ciliary epithelium[21]. AQP inhibition may thus provide a novel approach for the treatment of elevated IOP. The inhibitory effect of acetazolamide on AQP1 may be one of its mechanisms in reducing IOP.

Anordiol is considered as an antiestrogen, but there are evidences showing that this compound possesses potent agonist activity[22,23]. Anordiol caused water imbibition of uterine stroma and accumulation of uterine luminal fluid[22]. Li *et al* found that AQP1 gene expression in rat uterus was increased significantly 9 h after the last administration of three doses of anordiol (50, 250, 2500 μg/kg, sc for 3 d) respectively, and the stimulatory effect of anordiol was more pronounced than that of estrodiol, suggesting that production of water channels is the basis for the water imbibition of uterine cells[10]. The results in this study showed that after incubation for 15 min, anordiol did not affect the osmotic swelling of AQP1-cRNA injected oocytes, suggesting that anordiol did not directly inhibit the osmotic permeability of AQP1 that has been expressed in *Xenopus* oocyte membrane. But after incubation for 72 h, anordiol significantly inhibited the protein expression of AQP1 in *Xenopus* oocyte membrane, and appeared a dose-dependent manner. This is contrary to Li *et al*'s discovery in rat uterus, the causes may attribute to its dosage, tissue specificity or estrogen level in the body of animal. For example, in the immature rat anordiol produces estrogenic responses in the vagina, uterus and in the hypothalamic-pituitary-ovarian axis, but in mature rat the responses may be antiestrogenic[24]. Lu and Chatterton found that the presence of cornified cells in the vaginal smears 2 days after treatment with anordiol was a result of luteolysis and an intrinsic estrogenic activity of anordiol, but the uterine responses were characteristic of an antiestrogenic action of anordiol[22]. Whether anordiol is involved in the translation or translocation of AQP1 protein need to be clarified

So far now, there is an extensive body of information about aquaporin molecular genetics, tissue localization, developmental expression, molecular structure and function. It has been assumed that aquaporins are centrally important in mammalian physiology from aquaporin expression patterns and the high water permeability found in specific cell types. However, there is a paucity of direct investigation about the physiological significance of aquaporins, in part because there are no aquaporin inhibitors suitable for *in vivo* use.

The current results demonstrated that both acetazolamide and anordiol inhibited the osmotic water permeability of AQP1-cRNA injected oocyte, but their mechanisms were different. Acetazolamide functionally inhibited the osmotic water permeability of AQP1, whereas anordiol primarily decreased the amount of AQP1 protein expressed in the oocyte membrane. The present results implied that acetazolamide or anordiol might be validated as the potential compounds for the new therapeutic intervention in clinical disorders that associated with disturbances of aquaporin function.

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