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Expression of aquaporins in *Xenopus laevis* oocytes and glial cells as detected by diffusion-weighted ^{1}H NMR spectroscopy and photometric swelling assay

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Abstract

Expression of aquaporins (AQP) and water permeability were studied in *Xenopus laevis* oocytes and immobilized glial cells by a pulsed-field gradient spin echo NMR technique and a photometric swelling assay. Oocytes injected with poly(A) RNA from C6-BU-1 cells showed increased swelling behavior under hypoosmotic stress due to expressed water channels as compared to control oocytes. The swelling could be reversibly inhibited by $HgCl_2$. Furthermore, the intracellular relaxation time and the apparent intracellular diffusion coefficient of water in oocytes were determined by diffusion-weighted ^{1}H NMR experiments to be $T_2 = 36$ ms and $D_{app,intra} = 0.18 \times 10^{-3}$ mm^2/s. In immobilized C6 and F98 cells the mean exchange time of intracellular water was found to be 51 ms which increased to 75 ms upon chronic treatment (4 days) in hypertonic medium. Additional hybrid depletion experiments with antisense oligonucleotides directed against AQP1 were performed on oocytes and C6 cells. Moreover, different water channel subtypes of glial cells were assessed by a reverse transcriptase polymerase chain reaction assay. With this, the mRNA encoding AQP1 could be detected in primary cultures and glial cell lines, whereas AQP4 mRNA was found in astroglia-rich primary cultures, but not in F98 and C6 cells. Our results show that water permeability in glial cells is mainly mediated by water channels which play an important role in the regulation of water flow in brain under normal and pathological conditions.

Keywords: Aquaporin; Water exchange time; Hybrid depletion; RT-PCR; Oocyte; C6-BU-1 cell; F98 cell

Abbreviations: AQP, aquaporin; WCH, water channel; NMR, nuclear magnetic resonance; RT-PCR, reverse transcriptase polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RVD, regulatory volume decrease

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1. Introduction

Water channels (aquaporins, AQP) are selective pathways of water flow through biological membranes. They have been most intensively studied in erythrocytes and renal cells but seem to be present in almost all cell types [1–8]. So far five different types of water channels have been described in mammalian cells, numbered AQP1–AQP5 [9]. Water permeability through aquaporins is blocked by mercurials, e.g. $HgCl_2$ or *p*-chloromercuriobenzenesulfonate (pCMBS), except in AQP4, also known as the mercury-insensitive water channel (MIWC).

Two different water channels, AQP1 and AQP4, have been identified in brain [10–12]. AQP1 is mainly expressed in the choroid plexus, whereas AQP4 has been found in astrocytes and meningeal cells [11,12]. AQP4 channels are primarily located in the endfeet of astrocytes. There is good evidence that the well known orthogonal array particles of astrocytic endfeet are constituted by these water channels [12–14]. Aquaporins seem to play an important role in the maintenance of glial cell volume under normal and pathological conditions [15–17]. Swelling of astrocytes occurs especially under a variety of pathological states, e.g. ischemia, hypoxia, hypoglycemia or osmotic challenge. However, glial cells maintain an appropriate cell volume by adaptive volume regulatory mechanisms (regulatory volume decrease and regulatory volume increase) to prevent central nervous system dysfunction [18,19]. Functional differences among the aquaporins during volume regulation are to be expected as the single channel water permeability of AQP4 has been found to be fourfold higher compared to AQP1 [20,21].

Several methods have been developed to follow the movement of water through biological membranes. Changes in cell volume can be recorded by optical, electrical, and tracer methods [22]. The intracellular water exchange time and the permeability of the cell membrane are assessable by influx or efflux techniques (using e.g. deuterated or tritiated water [23–25] and spectroscopic fluorescence or NMR techniques based on different relaxation [26–31] or diffusion properties [32–36]). While the permeability properties have been often investigated in erythrocytes and other cells [26,30,32,33,37–39], there is only scarce information available regarding the membrane permeability of glial cells to water [36].

In the present work the expression of water channels was studied in glial cells (C6, F98, astroglia-rich primary cultures), neuron-rich primary cultures, and *Xenopus laevis* oocytes expressing mRNA isolated from C6-BU-1 glioma cells. *X. laevis* oocytes have been used in the past for the characterization of cloned water channels [40]. By manipulation of the membrane permeability to water one would expect that cell volume changes under anisosmotic conditions are affected. Therefore, the swelling behavior of *X. laevis* oocytes injected with poly(A) RNA from C6-BU-1 glioma cells under hypoosmotic conditions was monitored by diffusion-weighted ^1H NMR spectroscopy and a photometric swelling assay. The NMR methods made it possible to detect the intracellular water signal in oocytes and immobilized glial cells separately from extracellular water and to determine the intracellular water exchange time and changes of the intracellular volume directly. With hybrid depletion experiments directed against AQP1 and a reverse transcriptase polymerase chain reaction (RT-PCR) assay specific water channel subtypes of glial cells could be assessed.

2. Materials and methods

2.1. Oocytes and injections

X. laevis females were obtained from H. Kähler (Institut für Entwicklungsbiologie, Hamburg, Germany). The animals were anesthetized and ovarian lobes surgically removed using standard procedures. Oocytes were defolliculated by collagenase treatment (2 mg/ml) in OR2− buffer (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 5 mM HEPES, pH 7.8). Collagenase was removed carefully by washing cells several times with OR2−. Oocytes were incubated at 291 K overnight in OR2+ buffer (OR2− supplemented with 1.0 mM $CaCl_2$). Healthy looking stage V/VI oocytes (animal pole evenly colored, sharp border between both poles) were selected on the following day for injection. 50 nl of poly(A) RNA (approximately 1 ng/nl) or H_2O was injected with a microinjection device (Bachhofer, Reutlingen,

Germany). Oocytes were incubated for 1–6 days at 291 K in OR2+ containing gentamicin (10 mg/l).

2.2. Photometric swelling assay of the oocytes

Water permeability of oocytes was examined by exposure to hypoosmotic medium. Single water-injected and poly(A) RNA-injected oocytes were transferred into microcuvettes containing 1:5 diluted OR2+ buffer and were immediately placed in the two light paths of a double-beam spectrophotometer (Uvikon 860, Kontron Instruments, Munich, Germany). Swelling was assayed by increasing blockage of the light path, resulting in an increased light absorption at 600 nm. In all experiments the absorption difference was recorded between swelling of a water-injected oocyte and swelling of a poly(A) RNA-injected oocyte. The swelling reaction of three to five oocytes was recorded for each experiment.

2.3. Hybrid depletion

For hybrid depletion the oligonucleotide AQP1a with the sequence 5′ GAG GTG AGC ACC ACT GAT 3′, corresponding to bases 208–225 of the published rat AQP1 sequence (accession number X67948 or S49827), was used. The oligonucleotide was dissolved in 100 mM NaCl at a final concentration of 4 μg/μl. The poly(A) RNA was dissolved in water at a final concentration of 2 μg/μl. 5 μl of the oligonucleotide solution was mixed with 1.8 μl 1 M NaCl and 26.2 μl water. The poly(A) RNA was incubated at 65°C for 3 min. Subsequently 0.8 μl poly(A) RNA was mixed with 2 μl oligonucleotide solution. This mixture was incubated at 42°C for 10 min, then chilled on ice and immediately injected into oocytes. Each oocyte received an aliquot of 50 nl containing approximately 30 ng poly(A) RNA and 20 ng antisense oligonucleotide.

2.4. RT-PCR assay

2.4.1. RT reaction

RNA was isolated from rat astroglia-rich and neuron-rich primary cultures, F98 glioma cells and C6-BU-1 glioma cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method [41]. Poly(A) RNA was isolated from total RNA by two passages over an oligo(dT)-cellulose column [42]. Random hexamers (0.2 μg) were added to 1 μg poly(A) RNA in a total volume of 12 μl. The mixture was incubated for 10 min at 65°C and then chilled on ice. DTT, dNTPs and 5× reverse transcriptase buffer were added and the whole mixture (20 μl) was further incubated at 25°C for 2 min. For cDNA synthesis 200 μl of Superscript II-RT (Gibco BRL, Eggenstein, Germany) was added followed by incubation at 25°C for 10 min and then at 42°C for 1 h. Before PCR this mixture was inactivated by heating to 75°C for 15 min.

2.4.2. PCR

A standard PCR protocol with 100 pmol of each primer and a 2 μl aliquot of the heat-inactivated RT reaction was used for amplification of the fragments during 30 cycles (94°C 1 min; 50°C 1.5 min; 72°C, 2 min) in a Trio-Thermoblock using Primezyme Polymerase (Biometra, Göttingen, Germany). After amplification the samples were extracted once with chloroform and 20 μl was analyzed by electrophoresis through a 1.5% agarose gel. The following primers were used for AQP1: WCH3 (5′ GGT GCT CAC CTC AAC CCA 3′) corresponding to bases 274–292 and WCH4 (5′ TGA CCG GGC AGG GTT GAT 3′) corresponding to bases 631–649 of the published rat AQP1 sequence (accession number X67948 or S49827). AQP4 was detected with the oligonucleotides WCH5 (5′ ACG TCG CCT AAA GGA AGC 3′) corresponding to the sequence bases 777–795 and WCH6 (5′ AAC GCA CAT GAT TCG GTG 3′) corresponding to the sequence bases 1381–1399 of the published rat AQP4 sequence (accession number u14007). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected with the oligonucleotides GAPDH3 (5′ AAC TAC ATG GTC TAC ATG TT 3′) and GAPDH4 (5′ GTG GTG CAG GAT GCA TTG CT 3′) corresponding to bases 186–205 and 507–526, respectively, of the rat GAPDH sequence. Expression of GAPDH was used to check the efficiency of the RT reaction.

2.5. Preparation of the cell cultures and oocytes for the NMR experiments

The preparation of C6 and F98 rat glioma cells for the NMR experiments [43,44], the materials used,

pulse sequences, and the data analysis were performed as previously described [36,45–48]. Standard culture medium was Dulbecco's modified Eagle medium supplemented with 40 μM taurine and 5% fetal calf serum. For the NMR measurements, the cells were embedded in basement membrane gel (BMG) threads, transferred under sterile conditions into an 8 mm NMR tube with a perfusion insert and were perfused at 310 K with culture medium (1 ml/min) oxygenated with 95% O_2/5% CO_2. About 10^8 cells (determined by cell counting) were used in each experiment. An intracellular volume fraction of 0.05 was estimated based on an average cell diameter of 6–8 μm and a sample volume of 0.5 ml. The osmolarity of the media (hypotonic 180 mosm/l, isotonic 300 mosm/l, hypertonic 420 mosm/l) was modified by changing the NaCl concentration. The chronically treated cells were cultivated as usual, but using hypertonic medium or medium with antisense oligonucleotide (4.9 μmol/l) added during 4 days before the measurement.

In order to determine swelling of oocytes, about 50 oocytes were transferred into an 8 mm NMR tube and perfused with the OR2+ buffer at 291 K. The hypoosmotic conditions were – analogously to the photometric swelling assay – induced by a 1:5 dilution of the medium.

2.6. ^1H NMR spectroscopic monitoring of cell volume and water exchange time

The diffusion-weighted ^1H NMR experiments were performed on a Bruker AMX 360 MHz (8.4 T) system. The maximum available gradient strength with the 8 mm probe was 333 mT/m in the z direction. The diffusion-weighted spin echo signals were obtained by a 90°-τ-180°-τ sequence with unipolar gradients during τ. The experimental parameters determining the diffusion attenuation were the gradient duration δ, the gradient strength G and the separation Δ of the leading edges of the gradients, which defines the q value as $q=(\gamma\delta G)^2$, the diffusion time as $t_D=\Delta-\delta/3$, and the b value as $b=qt_D$. T_2 relaxation of the water signal could be examined by changing the RF pulse interval τ and the echo time $TE=2\tau$, respectively.

Three types of experiments with single signal (*ss*), constant diffusion time (*ct*), and constant gradient strength (*cg*) [36] were performed to observe the signal attenuation S of water dependent on G and Δ, i.e. the q value and the diffusion time. For *ss* experiments successive signals at a constant G and Δ were acquired. For *ct* experiments G was varied and Δ was kept constant, for *cg* experiments correspondingly Δ was varied and G kept constant. The signal intensity $S(G,\Delta)=S(q,t_D)$ was obtained by a Fourier transformation, an automatic phase correction of the spectra and an integration of the water peak.

ct experiments were performed at different echo times TE and diffusion times t_D to examine the intracellular water signal of oocytes (128 steps of G with a repetition time TR = 3 s, no averages). Apparent diffusion coefficients D_{app} were calculated from the negative slope of ln S vs. b value by linear regression (constant t_D). The transversal relaxation time T_2 was calculated for each b value from the negative reciprocal slope of ln S(TE) vs. TE (constant t_D). Relative changes of the intracellular water signal of oocytes and cells were monitored with *ss* experiments at one high b value (TR = 3.5–4 s), which can be regarded as proportional to changes of the intracellular volume.

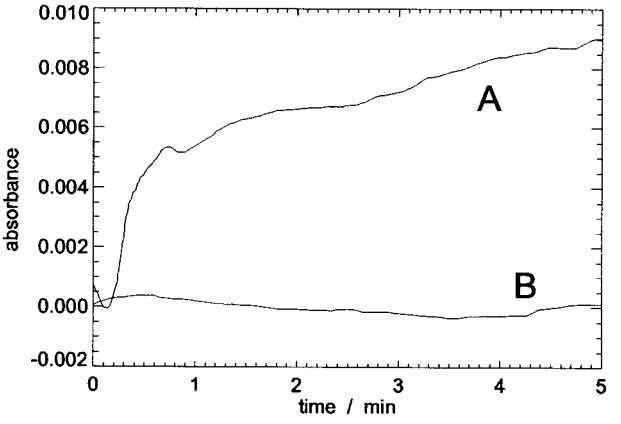

Fig. 1. Swelling of a poly(A) RNA-injected oocyte in hypotonic buffer and inhibition by $HgCl_2$. Oocytes were injected either with 50 ng poly(A) RNA (1 mg/ml), isolated from C6-BU-1 glioma cells, or with an equivalent volume of water. After incubation for 5 days in OR2+ buffer, expression of water channels was determined by a swelling assay. Single poly(A) RNA-injected and water-injected oocytes were placed in the light paths of a double-beam spectrophotometer. Swelling was initiated by a fivefold dilution of the OR2+ buffer with water. (A) The difference of light absorption between the poly(A) RNA-injected and the water-injected oocyte was recorded. (B) In a second experiment 0.3 mM $HgCl_2$ was added to the poly(A) RNA-injected and the water-injected oocyte.

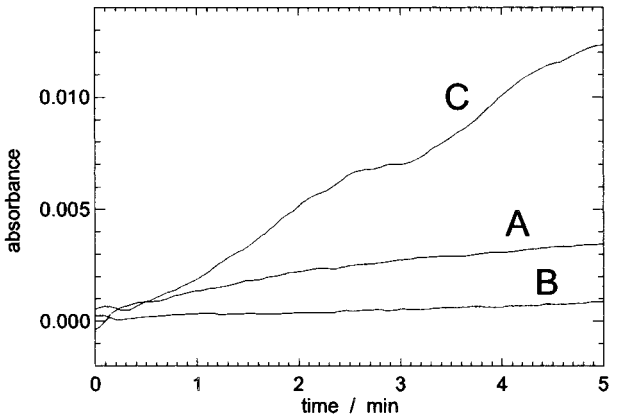

Fig. 2. Reversibility of $HgCl_2$ inhibition by 2-mercaptoethanol. Swelling of single poly(A) RNA-injected and water-injected oocytes was performed in hypotonic buffer and the difference in light absorption was recorded as described in Fig. 1. (A) Swelling in hypotonic OR2+ buffer. (B) Swelling in hypotonic buffer containing 0.3 mM $HgCl_2$. (C) Subsequent to the incubation in (B), 5 mM 2-mercaptoethanol was added to the hypotonic buffer and swelling was observed for five additional minutes.

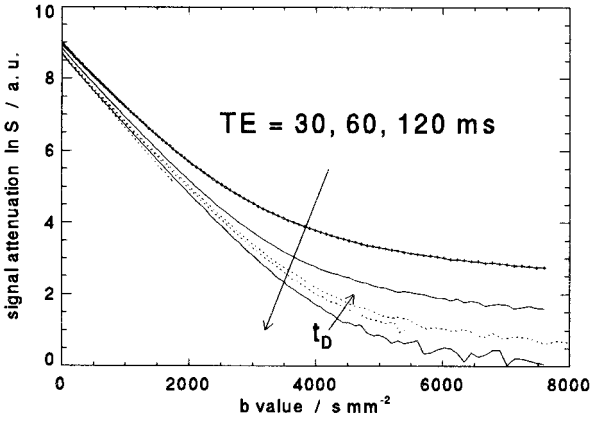

Fig. 3. Diffusion-attenuated ^1H NMR signal of water in *X. laevis* oocytes as measured by *ct* experiments with different echo times TE and diffusion times t_D ($\delta = 8$ ms, T = 291 K). The signal of the curves with the continuous lines decreased with increasing TE = 30, 60, 120 ms ($t_D = 15.3$ ms remained constant), whereas the signal of the dotted curves increased with increasing t_D=12.3, 37.3, 102.3 ms (while TE = 120 ms remained constant). Note: The data points acquired in the upper curve (TE = 30 ms) are marked with small cross symbols.

cg experiments at larger diffusion times $t_D > 50$ ms were used to determine the mean intracellular exchange time τ_{intra} for water in cell cultures (64 or 128 steps of Δ, constant TE = 120 s, TR = 3 s, no averages). τ_{intra} was calculated from the negative reciprocal slope of ln $S(\Delta)$ vs. t_D (constant q).

3. Results and discussion

3.1. Studies in X. laevis oocytes

3.1.1. Photometric swelling assay

Oocytes injected with poly(A) RNA from C6-BU-1 glioma cells burst after 20–30 min in initial experiments when exposed to hypotonic buffer, whereas water-injected oocytes were unaffected by the same treatment. To investigate this phenomenon more quantitatively a poly(A) RNA-injected and a water-injected oocyte were placed in cuvettes in the two beams of a spectrophotometer and the difference in absorption was recorded continuously. The poly(A) RNA-injected oocytes started to swell immediately after addition of hypotonic buffer, unlike the water-injected oocytes (Fig. 1, curve A). After 5–10 min extruded egg yolk from the bursting oocyte was visible in poly(A) RNA-injected oocytes but not in water-injected oocytes and the absorption increased strongly.

The swelling of the poly(A) RNA-injected oocyte

Table 1
Analysis of the *ct* experiments at different echo times TE and diffusion times t_D in Fig. 3[a]

(a)	TE (ms)	30	60	120	
	$D^{b,c}_{app,extra}$	1.89 ± 0.01	2.04 ± 0.02	2.14 ± 0.02	
	$D^{d}_{app,intra}$	0.176 ± 0.006	0.18 ± 0.02	0.22 ± 0.06	
(b)	t_D (ms)	12.3	15.3	37.3	102.3
	$D^{c}_{app,extra}$	2.18 ± 0.03	2.14 ± 0.02	2.05 ± 0.03	2.05 ± 0.02

[a]Apparent diffusion coefficients D_{app} were calculated by linear regression from the negative slope of ln S vs. b value.
[b]D_{app} calculated from signal at low and high b values were termed $D_{app,extra}$ and $D_{app,intra}$, as they origined mainly from extracellular and intracellular water, respectively.
[c]Evaluated in the range $b = 50$–200 s/mm^2.
[d]$b = 5900$–7600 s/mm^2.

could be completely suppressed by addition of 0.3 mM $HgCl_2$ to the diluted Ringer solution (Fig. 1, curve B), suggesting that $HgCl_2$-sensitive water channels were expressed in C6-BU-1 glioma cells. Inhibition by $HgCl_2$ was abolished in the presence of 2-mercaptoethanol, which shows that the effect of $HgCl_2$ is reversible (Fig. 2). The increased swelling response after treatment with 2-mercaptoethanol was not observed consistently in all experiments and is therefore not considered significant.

3.1.2. Diffusion-weighted ^1H NMR spectroscopy

The properties of intracellular water in poly(A) RNA-injected oocytes were studied by ^1H NMR methods. *ct* experiments were performed on untreated oocytes at different echo times TE and diffusion times t_D (Fig. 3). The diffusion-attenuated signal curves of water decreased with increasing TE = 30, 60, 120 ms at constant $t_D = 15.3$ ms (continuous lines). The appertaining relaxation time $T_2 = 313 \pm 19$ ms was calculated at low b values (b = 0–110 s/mm^2). It decreased continuously and remained constant at $T_2 = 36 \pm 2$ ms for $b > 4500$ s/mm^2 (average in the range b = 4500–7600 s/mm^2). In contrast, the water signal increased with increasing diffusion time t_D = 12.3, 37.3, 102.3 ms at constant TE = 120 ms (dotted lines) as indicated by the short arrow in Fig. 3.

To analyze the *ct* curves in more detail, apparent diffusion coefficients D_{app} and their standard deviations were calculated from the negative slopes of the curves as shown in Table 1. D_{app} at low and higher b values was termed $D_{app,extra}$ and $D_{app,intra}$ assigning the signal mainly to extra- and intracellular water, respectively. When a part of the intracellular water encounters restrictions in diffusion, e.g. by membranes, etc., the mean displacement of the molecules in the average is lowered and reduces the apparent diffusion coefficient D_{app} of this compartment up to two orders of magnitude [36,48]. Additionally, D_{app} decreases with increasing diffusion time. In our system, the NMR signal at higher b values is determined mostly from water of these compartments, where restriction is evident from the lowered D_{app}.

$D_{app,extra}$ increased from 1.89 to 2.14×10^{-3} mm^2/s with increasing TE and $D_{app,intra}$ from 0.18 to 0.22×10^{-3} mm^2/s. With increasing t_D, however, $D_{app,extra}$ decreased from 2.18 to 2.05×10^{-3} mm^2/s,

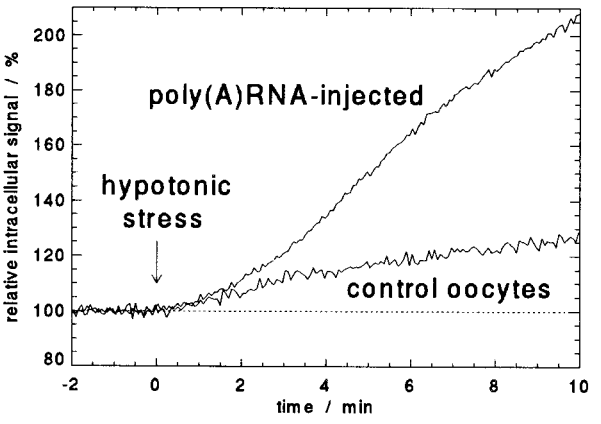

Fig. 4. Swelling of poly(A) RNA-injected oocytes in hypotonic buffer as detected by diffusion-weighted ^1H NMR. About 50 poly(A) RNA-injected oocytes were prepared as described in the legend of Fig. 1. Successive signals of intracellular water were acquired at b=5400 s/mm^2 with a repetition time of TR = 4 s (TE = 36 ms, t_D = 22.3 ms, T = 291 K). A minor swelling upon hypotonic stress was observed with the control oocytes (~50, water-injected).

and is close to the diffusion constant of free water $D = 1.91 \times 10^{-3}$ mm^2/s at 291 K [49]. Because of experimental limitations in the gradient strength, $D_{app,intra}$ could not be measured and evaluated quantitatively at all diffusion times t_D = 12.3–102.3 ms. However, $D_{app,intra}$ indeed showed a tendency to decrease with t_D.

The swelling of poly(A) RNA-injected oocytes under hypotonic stress was detected with ^1H NMR *ss* monitoring in Fig. 4 (b = 5400 s/mm^2, TE = 36 ms, t_D = 22.3 ms). The intracellular water signal could be acquired separately from extracellular signal contributions at high b values as shown by the *ct* experiments. The relative intracellular water signal of the poly(A) RNA-injected oocytes increased by more than 100% within 10 min in the hypotonic medium, whereas the signal of the control oocytes increased by 25% only. The standard deviation of the relative signal intensity under control conditions was lower than 2% which stated the high sensitivity for the detectable changes in the intracellular water signal.

Different biological surroundings of water have been discussed before [50], and distinct water or hydration compartments have been found in *X. laevis* oocytes discriminating hydrogen bounding mechanisms and NMR relaxation properties in the nucleus and cytoplasm [51–53]. Our *ct* experiments on the

oocytes agree in a twofold manner regarding the properties of intracellular water.

(1) *Diffusion properties*: The observed decrease of the apparent diffusion coefficient of about one order of magnitude at larger diffusion weighting is explained by the restricted diffusion of intracellular water compartments in contrast to the free diffusion of extracellular bulk water [48].

(2) *Relaxation properties*: The NMR T_2 relaxation times have shown the same characteristic and have reached a constant $T_2 = (36 \pm 2)$ ms at high b values which can be regarded as an average intracellular T_2. On the other hand, the water signal at low b values can be regarded as a superposition of the volume-weighted signal contributions of the bulk and the intracellular water.

Recent NMR microscopical measurements have shown local differences of T_2 in oocytes, i.e. $T_2 = 11$, 19, 29 ms in the nucleus, and in the cytoplasm of the animal pole and the vegetal pole, respectively [52]. Signals from compartments with shorter T_2 had decayed and were no longer detectable at the larger TE used in our experiments. The assignment of the intracellular signal to an individual intracellular compartment remains problematic and is not evident from our data. Considering this, the detected changes of the intracellular signal at high b values under osmotic stress (Fig. 4) can probably not be interpreted as the quantitative volume change of the oocytes. Possibly, the properties of the intracellular water pools (e.g. yolk platelet and non-yolk cytoplasm) are altered during the oocyte swelling [51] and thereby the contributions to the detected water signal are changed. Relative changes of the intracellular water signal between the treated and the control oocytes are therefore more reliable.

3.2. Characterization of glial water channels

3.2.1. Hybrid depletion

Only two types of water channels, AQP1 and AQP4, have been detected in brain cells so far [10–12]. The $HgCl_2$-insensitive AQP4 has been reported to be the principal water channel of glial cells, whereas expression of AQP1 is restricted to the choroid plexus [11,12]. The inhibition of the oocyte swelling by $HgCl_2$ as seen in Figs. 1 and 2, however, strongly indicated the presence of a mercury-sensitive water channel in C6-BU-1 glioma cells. In order to investigate whether swelling of the oocytes indeed resulted from expression of glial water channels, hybrid depletion experiments were performed. Coinjection of antisense oligonucleotides targeted against the AQP1 mRNA together with poly(A) RNA from C6-BU-1 cells completely suppressed swelling of poly(A) RNA-injected oocytes in response to hypotonic buffer (Fig. 5).

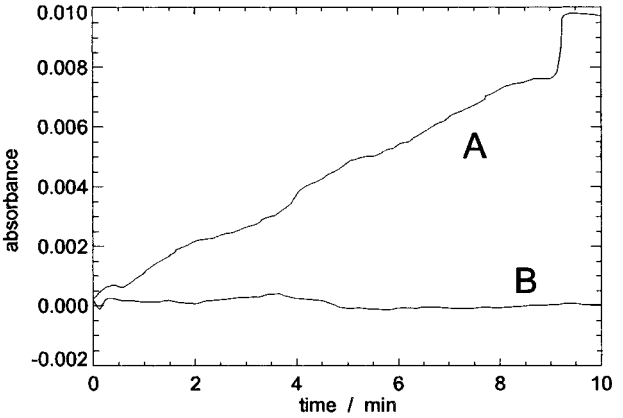

Fig. 5. Hybrid depletion experiments with antisense oligonucleotides directed against AQP1. Oocytes were injected either with 30 ng C6-BU-1 glioma cell poly(A) RNA (in 50 nl), 30 ng C6-BU-1 glioma cell poly(A) RNA plus 20 ng oligonucleotide (in 50 nl) or 50 nl of water. Expression of water channels was recorded as described in Fig. 1. (A) Difference in swelling between a poly(A) RNA-injected oocyte and a water-injected oocyte. (B) Difference in swelling of an oocyte injected with poly(A) RNA plus antisense oligonucletide and a water-injected oocyte.

3.2.2. RT-PCR

In agreement with the oocyte experiment, the mRNA encoding AQP1 could be detected by RT-PCR, whereas AQP4 mRNA was not found in C6 glioma cells and only in small amounts in F98 cells and neuron-rich primary cultures (Fig. 6). The AQP4 mRNA found in neuron-rich primary cultures could be derived from contaminating astroglial cells present in these cultures. In primary cultures of astroglial cells both, mRNA for AQP1 and for AQP4, could be detected in similar amounts (Fig. 6). Although C6 glioma cells are often regarded as a cell line with astroglial properties, it has been shown for glutamate and glycine transporters that C6 glioma cells express different isoforms as astrocytes in culture or in adult brain [54,55]. Whereas the expres-

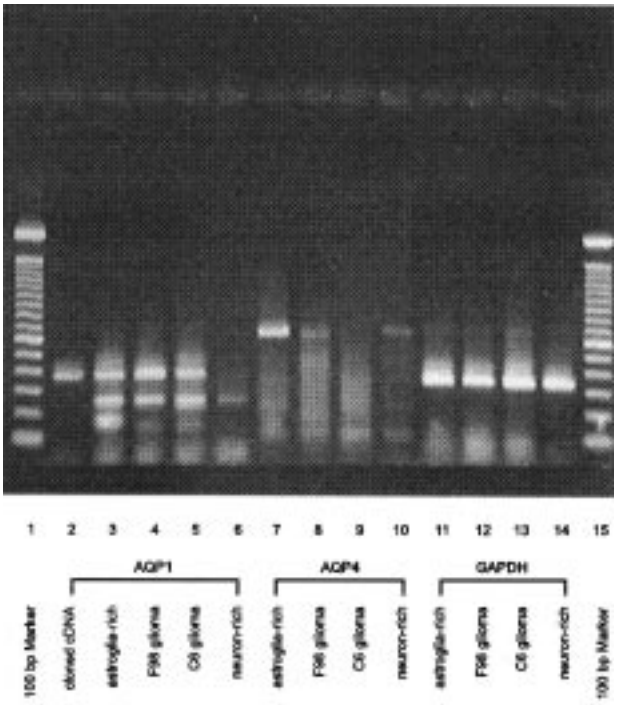

Fig. 6. RT-PCR detection of different water channel subtypes in glial cells. Messenger RNA was isolated from different cell types and subjected to RT-PCR using oligonucleotides specific for AQP1 (oligonucleotides WCH3 and WCH4, lanes 2–6), for AQP4 (oligonucleotides WCH5 and WCH6, lanes 7–10), and for GAPDH (GAPDH1 and GAPDH2, lanes 11–14). Nucleotides used: 100 bp marker (starting from 100 bp, lanes 1, 15), plasmid with cloned AQP1 cDNA (lane 2), rat astroglia-rich primary cultures (lanes 3, 7, 11), F98 glioma cells (lanes 4, 8, 12), C6 glioma cells (lanes 5, 9, 13), and neuron-rich primary cultures (lanes 6, 10, 14).

sion of AQP4 in astrocytes agreed with results from adult brain, AQP1 has not been detected in adult brain using immunohistochemical methods [10,12]. The cultured astrocytes used in this study were derived from neonatal brains and therefore proliferated rapidly after preparation, which might have favored the expression of AQP1. Some unspecific fragments were amplified in the PCR reaction for AQP1. Sequencing revealed that only fragments having the same size as the control reaction (Fig. 6, lane 2) corresponded to the mRNA of AQP1.

3.3. Volume regulation and water exchange time in glial cells during treatment with antisense oligonucleotides and chronic hypertonicity

Immobilized C6 and F98 glioma cells embedded in BMG threads were used to examine the effects of altered membrane permeability on the intracellular water signal and on water exchange by diffusion-weighted ^{1}H NMR spectroscopy. As the experiments performed on oocytes suggest, antisense oligonucleotides should be able to suppress the expression of aquaporins.

3.3.1. Treatment with antisense oligonucleotides

No alterations of the initial swelling behavior were detected on C6 cells during hypotonic stress which had been treated 4 days with antisense oligonucleotides. The swelling pattern and the subsequent regulatory volume decrease (RVD) were similar to Fig. 7 and previously published data of F98 cells (cell swelling in hypotonic medium) [36]. The water exchange time of C6 cells treated with antisense oligonucleotides ($n=2$) was determined by *cg* experiments to $\tau_{intra} = 67.9 \pm 0.8$ ms (range $t_D = 50$–95 ms, $q = 359 \times 10^3$ mm^{-2}). For untreated C6 cells τ_{intra} was 67.2 ± 3.3 ms.

3.3.2. Chronic hypertonicity

We examined whether chronic treatment in hypertonic medium (4 days before measurement) influences the extent of expressed aquaporins as a possible regulation mechanism due to altered conditions. The

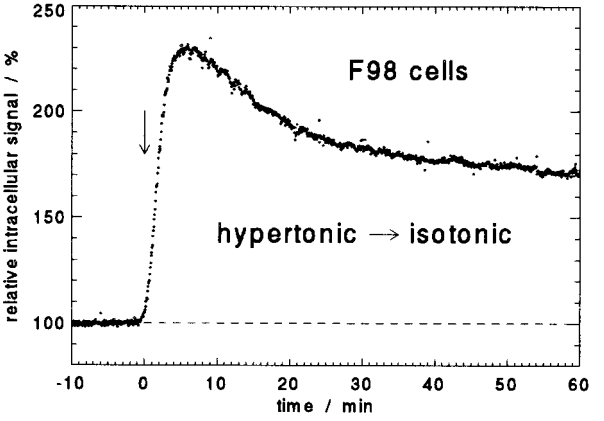

Fig. 7. Swelling of F98 cells (treated for 4 days in hypertonic medium) during osmotic stress induced by an isotonic medium. The intracellular water signal was monitored by a diffusion-weighted ^{1}H NMR *ss* experiment at $b = 15\,300$ s/mm^2 (TR = 3.5 sec, TE = 60 ms, $t_D = 31.7$ ms, T = 310 K). After the rapid initial swelling, a regulatory volume decrease was observed which was comparable with the normal situation of isotonically grown cells undergoing osmotic swelling induced by a hypotonic medium [36].

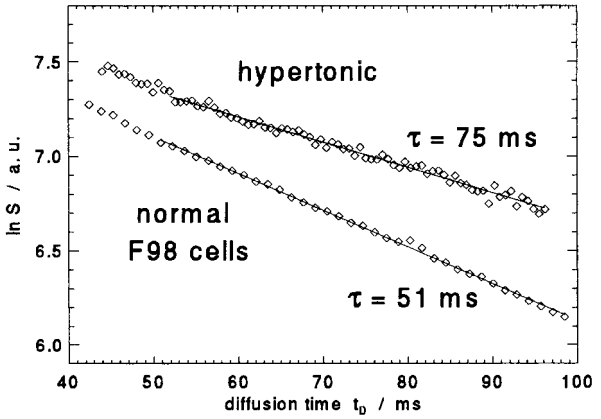

Fig. 8. Intracellular exchange time of water τ_{intra} in normal and chronically hypertonic treated F98 cells (4 days) as assessed by diffusion-weighted ^{1}H NMR *cg* experiments. Using high gradient strengths (q values), mainly the intracellular water signal was detected and τ_{intra} was calculated from the negative reciprocal slope of ln S vs. diffusion time. The chronically hypertonic treated cells showed an about 50% increased τ_{intra} compared to cells grown in isotonic medium. Experimental parameters: $q = 183 \times 10^3$ mm^{-2} (normal)/$q = 199 \times 10^3$ mm^{-2} (hypertonic), TE = 120 ms, TR = 3 s.

swelling pattern of F98 cells was monitored by a *ss* experiment during changing the hypertonic control medium to an isotonic medium (Fig. 8). Swelling in the ensemble was achieved within 3–4 min which is the same time as observed in normally grown F98 cells [36]. Also a typical two-step RVD of the relative intracellular water signal from 230 to 190% within 15–20 min and to 170% within 60 min was observed. The standard deviation of the relative signal intensity under control conditions was lower than 1%. The intracellular exchange time of water in normal and chronically hypertonic treated F98 cells was determined by *cg* experiments from the negative reciprocal slope of ln S vs. t_D in the range $t_D = 52$–96 ms (hypertonic medium) and $t_D = 51$–99 ms (control) in Fig. 8. The chronically hypertonic treated cells showed an approximately 50% increased mean intracellular water exchange time $\tau_{intra} = 74.6 \pm 1.0$ ms compared to cells grown up in isotonic medium ($\tau_{intra} = 51.3 \pm 0.4$ ms).

It was found previously that during osmotic swelling and shrinkage neither the apparent diffusion coefficient at $b > 15\,000$ s/mm^2 (as detected by a *ct* experiment) nor the mean apparent intracellular exchange time (as detected by a *cg* experiment) changed within experimental error [36,48]. On the other hand no significant changes in cell size and cell shape could be observed by light microscopy during chronic hypertonic treatment for several days due to the regulatory volume increase performed by the cells. This suggests that the increased intracellular water exchange time correlates with a decreased membrane permeability, which could be explained by an altered expression of water channels, stimulated by the hypertonic treatment. For comparison, a dynamic increase of τ_{intra} from 51.9 ± 1.0 ms to 59.0 ± 1.1 ms has previously been observed with *cg* monitoring during treatment of perfused F98 cells with 0.5 mM pCMBS which is a well known blocker of mercury-sensitive water channels [36]. Moreover, the specific regulation of membrane water permeability in normal physiology and disease states as well as the regulation of gene expression by hypertonicity has been addressed in Refs. [56,57].

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