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Regulation of AQP-4 Water Channel Expression in the Brain during Development and by Ischemia

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Water transport is mediated by two distinct pathways, diffusional and channel-mediated water transport. The first molecular water channel was identified from human erythrocytes in 1992. Genetically-related proteins from other mammalian tissues have subsequently been identified to transport water, and the group is referred to as th "Aquaporins". Aquaporin-4 (AQP4) is most abundant in the brain, which may be involved in CSF reabsorption and osmoregulation. However, ontogeny and regulatory mechanisms of AQP4 channels have not been reported. Northern blot analysis showed that AQP4 mRNA began to be expressed in the brain just before birth and that its expression gradually increased by PN7 and then decreased at adult level. AQP4 was expressed predominantly in the ependymal cells of ventricles in newborn rats. And then its expression decreased in ependymal cells and increased gradually in other regions including supraoptic and paraventricular nuclei. AQP4 is also expressed in the subfornical organ, in which the expression level is not changed after birth. Cryogenic brain injury did not affect expression of AQP4 mRNA, while ischemic brain injury decreased it. Osmotic water permeability of AQP4 channel expressed in *Xenopus* oocytes was inhibited by the pretreatment of BAPTA/AM and calmidazolium, a Ca^{2+}/Calmodulin kinase inhibitor, in a dose-dependent manner. These results indicate that the expression and the function of AQP4 channel are regulated by developmental processes and various pathophysiological conditions. These results will contribute to the understanding of fluid balance in the central nervous system and the osmoregulatory mechanisms of the body.

Key Words: Aquaporin-4, Regulation, Brain, Ischemia

INTRODUCTION

Water transport is mediated by two distinct pathways, diffusional and channel-mediated water transport (Finkelstein, 1987). The aquaporins are a family of water-selective membrane channels found in animals, plants, and microorganisms (Agre et al, 1993; Knepper, 1994). Aquaporin-1 (AQP1, also known as CHIP, channel-forming integral membrane protein of 28 kDa) was the first protein shown to function as a molecular water channel (Preston et al, 1992) and is naturally expressed in mammalian red cells, renal proximal tubules, and other water permeable epithelia (Denker et al, 1988; Nielsen et al, 1993a; Nielsen, 1993b). AQP2 is the vasopressin-regulated water channel in renal collecting ducts (Fushimi et al, 1993) and is the site of mutations in some forms of nephrogenic diabetes insipidus (Deen et al, 1994). AQP3 is the water channel in the basolateral membranes of renal medullary collecting duct (Ishibashi et al, 1993).

Recently, aquaporin-4 (AQP4 or mercurial-insensitive water channel (MIWC)) has been cloned (Hase-

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gawa et al, 1994; Jung et al, 1994), of which mRNA was most abundant in the brain (Jung et al, 1994). Although AQP4 may contribute to the pathophysiology of normal pressure hydrocephalus, pseudotumor cerebri, or postischemic edema (King & Agre, 1996), such linkages have not been established. Jung et al (1994) reported that expression of AQP4 mRNA can be regulated developmentally. AQP4 possesses several consensus protein kinase phosphorylation sites (Hasegawa et al, 1994; Jung et al, 1994). However, changes in the distribution of AQP4 in the brain during development, changes in its expression under various pathophysiological conditions, and regulation of AQP4 channel by protein phosphorylation have not been examined. Therefore, this study was undertaken to determine changes in the expression of AQP4 mRNA during development and some pathophysiological conditions and regulation of AQP4 channel by protein phosphorylation in *Xenopus* oocytes.

METHODS

Animal preparation.

Male Sprague Dawley rats were used in the experiment. Animals were fasted overnight before surgery and had free access to water. Middle cerebral artery distribution ischemia was produced by the method of Zea Longa et al (1989). Briefly, animals were anesthetized with 3.5% halothane, and anesthesia was maintained with 0.5 to 1.0% halothane in 70% N_2O and 30% O_2, using a face mask. A 4~0 surgical nylon filament was advanced from the external carotid artery into the lumen of the internal carotid artery until it blocked the origin of the middle cerebral artery (MCA). After the intended period of ischemia, the filament was removed and the anesthesia discontinued during the period of recirculation. To make cyrogenic injury in the rat brain, a funnel was attached to the skull over the left hemisphere. Liquid nitrogen was poured into the funnel for precisely 90s. Water content of brain was measured by subtracting dry weight from wet weight of brain tissues.

In situ hybridization of brain

Cryosections were cut 12 mm thick from rat brains, fixed with 4% paraformaldehyde in phosphate-buffered saline, and treated with 0.25% acetic anhydride/0.1 M triethalonamine for 10 min. [α-[^{35}S]thio]-UTP-labeled antisense and sense RNA probes were made with T7 or T3 RNA polymerase from a linearized plasmid containing the AQP4 cDNA. Sections were hybridized overnight at 56°C with probe (10^6 cpm/ml) (Jung et al, 1994). After RNase treatment, the sections were exposed to autoradiographic film for 1 day and then to photograpic emulsion for 4 days.

Northern analysis

Total RNAs from brains were isolated using RNazol B solution. For Northern blot analysis, 10 μg of total RNA were separated in formaldhyde/1.2% agarose gels and transferred to nylon membranes (Genescreen plus, NEN). Blots were prehybridized at 65°C with denatured Herring sperm DNA (0.1 mg/ml) in 6×SSC, 2× Denhardt's solution and 0.5% SDS and hybridized at 65°C overnight in the same solution containing ^{32}P-labeled probe (10^6 cpm/ml) which was made by random primer labeling (Boehringer Manheimm) of the rat AQP4 cDNA. After washing, the blots were exposed 1~3 days to Amersham film at −70°C with intensifying screens. The resulting images and the 28S ribosomal RNA bands were quantitated by a image analysis system (Biorad) and the northern signals were normalized by the corresponding 28S ribosomal RNA.

in Vitro RNA Synthesis

Capped RNA transcripts were synthesized in vitro, and the RNA was purified as described (Yisraeli and Melton, 1989). RNA was synthesized with T3 RNA polymerase using Xba I-digested AQP4 expression vector DNA or AQP-CHIP expression vector DNA.

Preparation of oocytes and Measurement of osmotic water permeability (P_f)

Stage V and VI oocytes were removed from female *Xenopus laevis* and prepared as described (Lu et al, 1990) with the exception that the amphibia were anesthetized on ice. The day after isolation, oocytes were injected with either 50 nl of water or 50 ng of RNA in 50 nl of water. Injected oocytes were main-

tained for 3 days at 18°C in modified Barth's buffer prior to osmotic swelling (Preston et al, 1992). Modified Barth's solution was composed of (in mM) 88 NaCl, 1 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 2.4 $NaHCO_3$, and 10 HEPES (pH 7.4). Osmotic swelling at 22°C was monitored with a Olympus phase-contrast microscope equipped with a video camera connected to a computer. Oocytes were transferred from 200 mOsM (osm_{in}) to 70 mOsM (osm_{out}) modified Barth's buffer diluted with water. An oocyte image was captured by computer and stored at 15-s intervals for a total of 3 min or until the time of oocyte rupture. The surface area of the sequential images was calculated using assuming that the oocytes are spheres without microvilli. The oocyte volume was calculated using the following formula.

$$V=(4/3)\times(area)\times(area/\pi)^{1/2} \quad \text{(Eq. 1)}$$

The change in relative volume with time, $d(V/V_o)/dt$, up to 5 min (or time of oocyte rupture) was fitted by computer to a quadratic polynomial, and the initial rates of swelling were calculated. The osmotic water permeabilities (P_f, cm/s$\times 10^{-4}$) were calculated from osmotic swelling data between 15 and 30 s, initial oocyte volume ($V_o=9\times10^{-4}$ cm^3), initial oocyte surface area (S= 0.045 cm^2), and the molar ratio of water (V_w=18 cm^3/mol) (Jung et al, 1994) using the following formula.

$$P_f = [V_o\times d(V/V_o)/dt]/[S\times V_w\times(osm_{in} - osm_{out})] \quad \text{(Eq. 2)}$$

RESULTS

Developmental regulation of AQP4 mRNA expression

To evaluate developmental changes in the expression of AQP4 mRNA, total RNAs isolated from total brains of embryonic day (E) 17, E21, postnatal day (PN) 1, PN7 and PN60 rats were analyzed by Northern blot. Rat brains at E17 did not express AQP4 mRNA and the message began to be detected at E21. The expression of AQP4 mRNA gradually increased by PN7 and then decreased at adult level (Fig. 1).

Developmental changes in distribution of AQP4 mRNA was analyzed by *in situ* hybridization. AQP4 mRNA was expressed predominantly in ependymal cells of ventricles at PN1 and PN5. However, the expression of AQP4 mRNA in ependymal cells of ventricles decreased, while other regions of brain

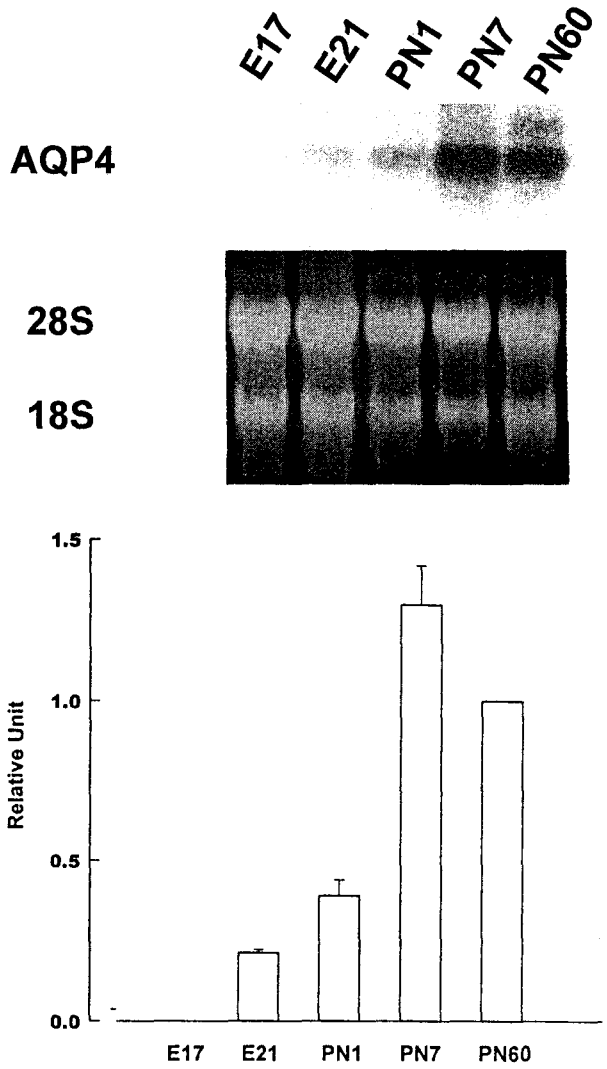

Fig. 1. The developmental expression of AQP4 mRNA by northern blot. Equal amounts (10 μg) of total RNA isolated from brains of E17, E21, PN1, PN7 and PN60 rats were electrophoresed on formaldehyde gel, transferred to nylon membrane and hybridized with rat AQP4 cDNA probe. Intact 28 S and 18 S ribosomal RNA bands are shown by ethidium bromide staining of agarose gel. The lower pannel represents normalized expression levels of AQP4 mRNA. To qunantitate the message level, the ratio of the northern signals to intensity of the corresponding 28S ribosomal RNA bands was calculated. Data were expressed as ratio to the value of PN60 rats (n=3).

including paraventricular and supraoptic nuclei increased in adult rats. However, expression of AQP4 mRNA in the subfornical organ did not change during development (Fig. 2). Higher magnification of emulsion autoradiograph of PN5 rats showed that AQP4 mRNA is strongly expressed at ependymal cells lining ventricles and not in the choroid plexus (Fig. 3).

Changes in AQP4 expression after ischemic and cryogenic brain injury

AQP4 mRNA is widely distributed in the brain (Jung et al, 1994). To examine the changes in AQP4 expression by brain injury, cryogenic and ischemic brain injuries were induced. To monitor the extent of brain edema after brain injury, the changes in water content of injured regions of brain were determined. Fig. 4 represents changes in water content of the brain by cryogenic brain injury. After cryogenic brain injury water contents in the brain increased gradually

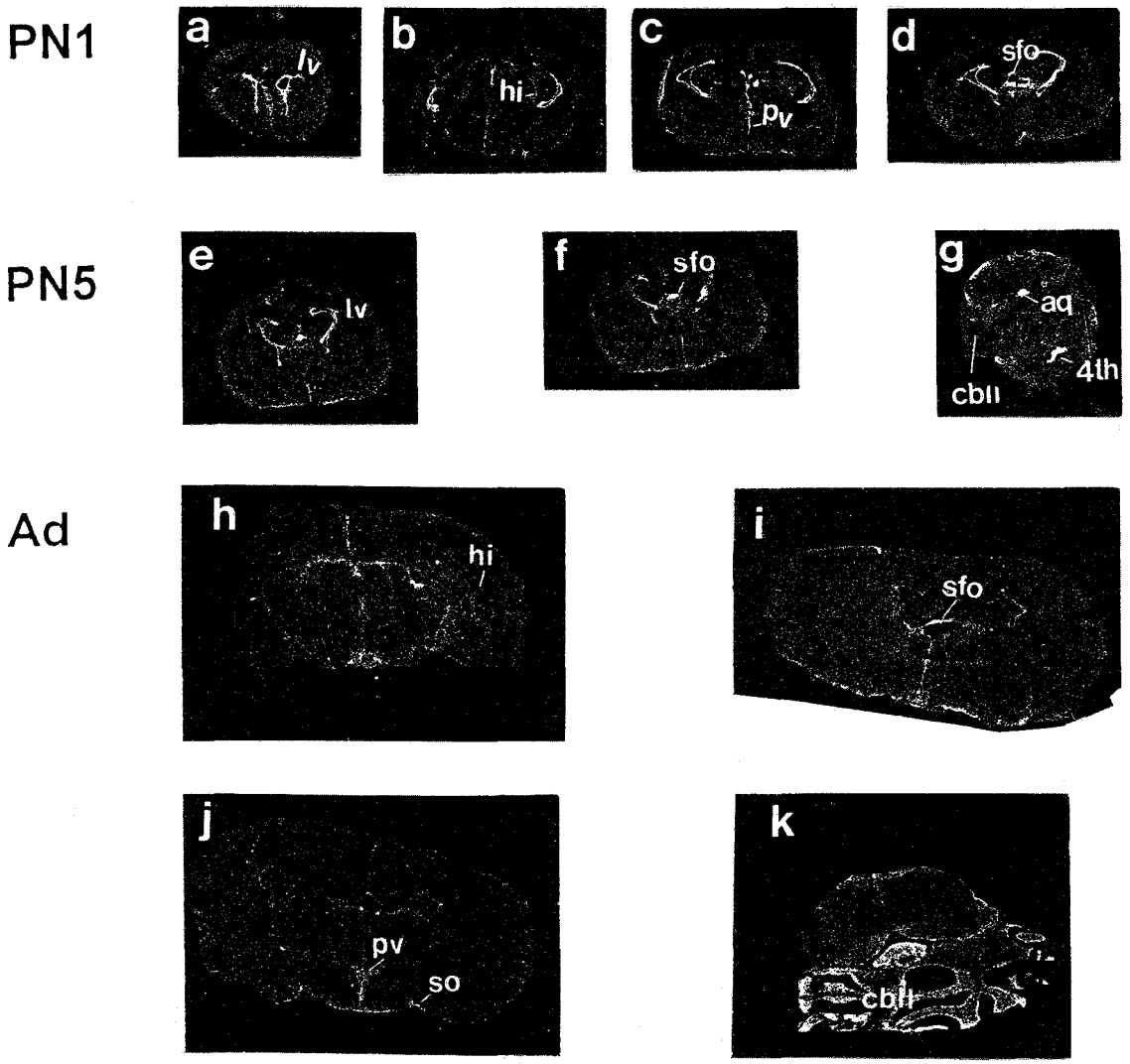

Fig. 2. The developmental distribution of AQP4 mRNA analyzed by *in situ* hybridization. The brain tissues obtained from 1-day-old (PN1), 5-day-old (PN5) or 2 month old (Ad) rats were hybridized with antisense AQP4 cRNA probe. lv, lateral ventricle; hi, hippocampus; pv, paraventricular nuclei; sfo, subfornical organ; aq, cerebral aqueduct; 4th, 4th ventricle; cbll, cerebellum, so, supraoptic nuclei.

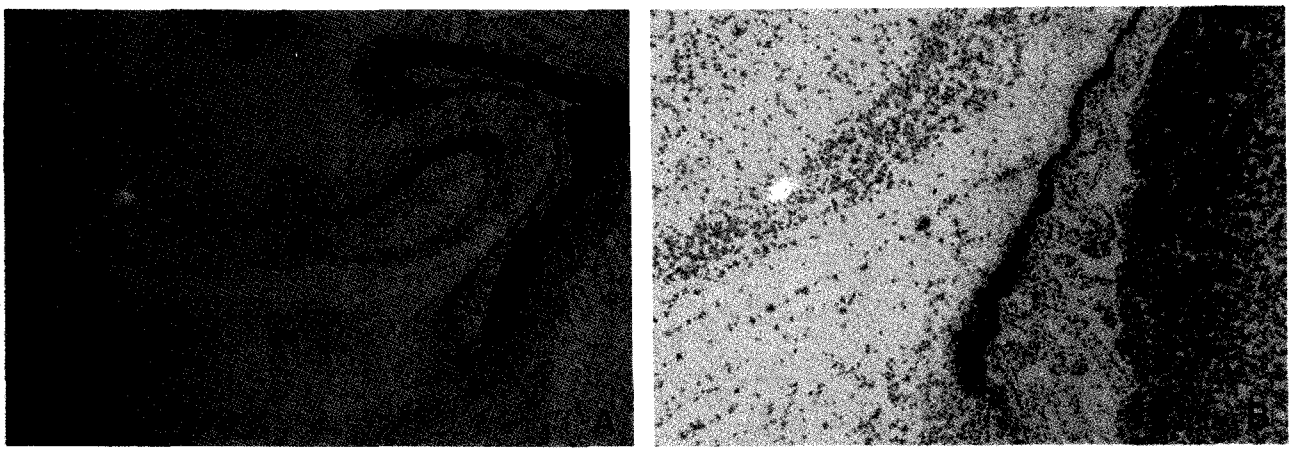

Fig. 3. Microscopic AQP4 mRNA localization by nuclear emulsion autoradiography from rat brain. Positive hybridization is shown by small dark granules. Final magnification A: ×50; B: ×125. Higher magnification clearly showed that ependymal cells in the lateral ventricles are hybridized and choroid plexus is not.

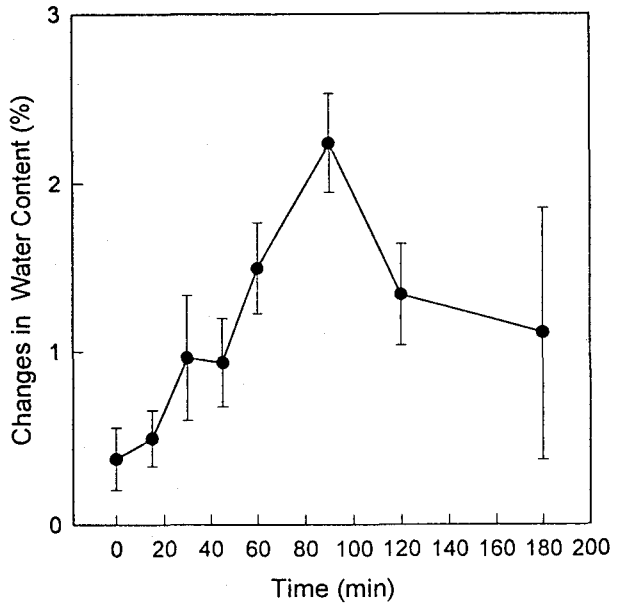

Fig. 4. Changes in the water content of rat brain by cyogenic brain injury. Brains were isolated at the indicated times after cryogenic injury, and water content was measured by the differences of weight between wet tissues and dry tissues. Data represent mean ± SEM(n=6).

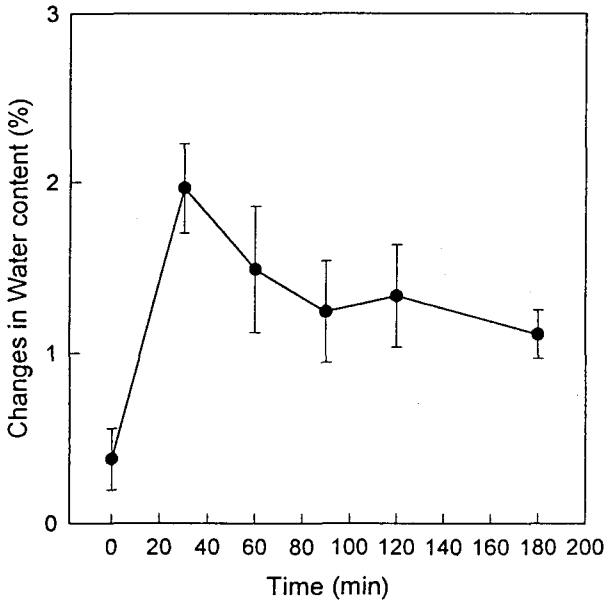

Fig. 5. Changes in the water content of rat brain by ischemic brain injury. Brains were isolated at the indicated times after 30 min occulsion of middle cerebral artery, and water content was measured by the differences of weight between wet tissues and dry tissues. Data represent mean ± SEM (n=5).

and reached the maximum swelling at 90 min after injury. Fig. 5 represents changes in water content of rat brain by ischemic brain injury. After ischemic brain injury water contents in the brain increased gradually and reached the maximum swelling at 30 min after injury.

The changes in AQP4 mRNA expression under these conditions were detemined by northern blotting analysis. Expression of AQP4 mRNA did not change by 6 hrs after cryogenic injury. However, AQP4 expression in ischemic brain was reduced greatly by

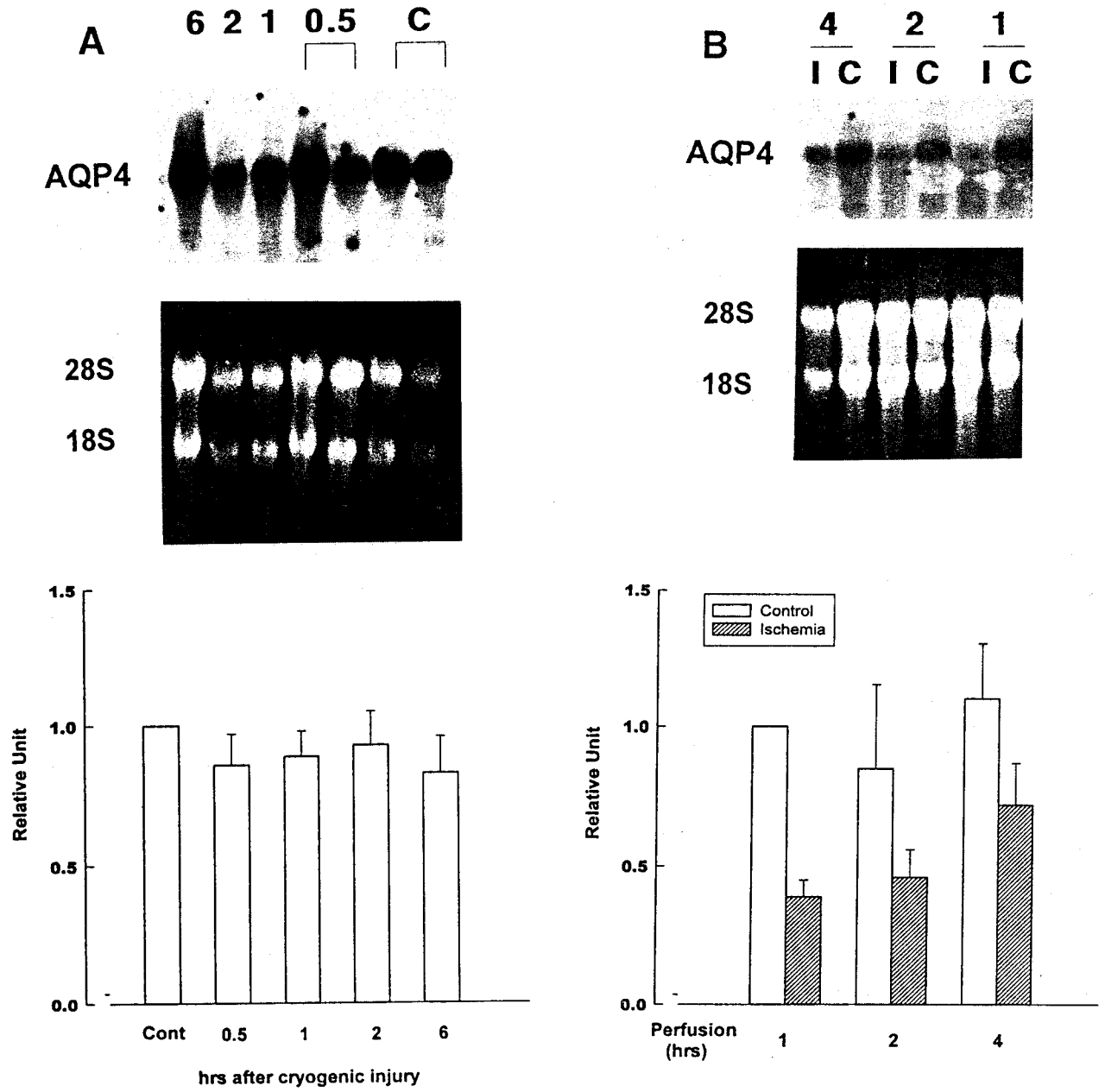

Fig. 6. Northern analysis of AQP4 mRNA in cryogenic and ischemic brain injury. A Total RNA was isolated from brains at the indicated times (hours) after cryogenic insult. B. Total RNA was isolated from brains (I) at the indicated times (hours) after reprefusion of the occluded middle cerebral artery. Control RNA (C) was isolated from the contralateral hemisphere of the same animals. Total RNA (10 μg) was analyzed by Northern blot. Intact 28S and 18S ribosomal RNA bands are shown by ethidium bromide staining of agarose gel. The lower pannel represents normalized expression levels of AQP4 mRNA. To quantitate the message level, the ratio of the northern signals to intensity of the corresponding 28S ribosomal RNA bands was calculated. Data were expressed as ratio to the value of control rats (A) and (B) control RNA isolated from rats which were perfused for 1hr after ischemia (n=3).

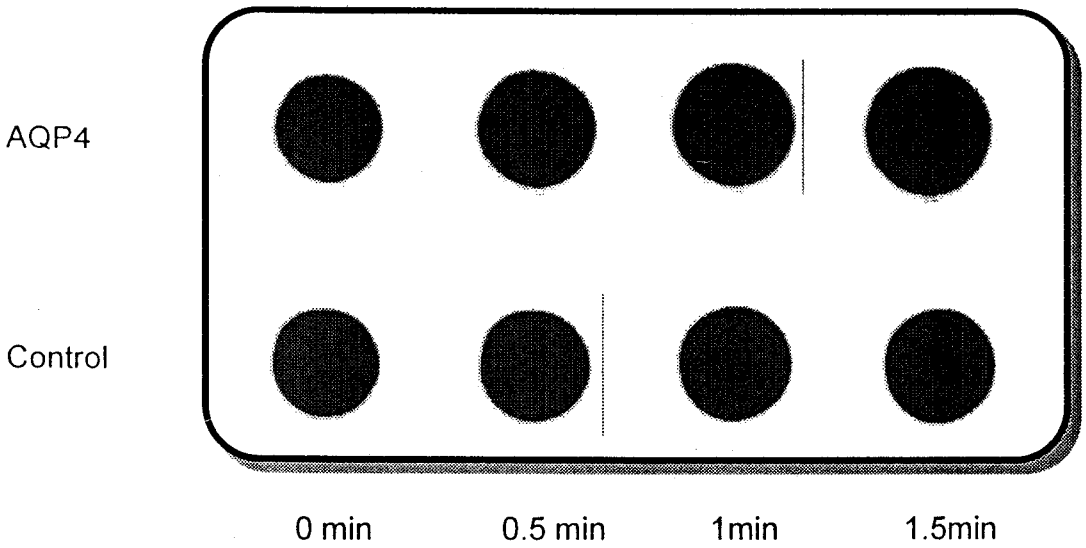

Fig. 7. Functional expression of channel-mediated water transport in *Xenopus* oocytes. AQP4 cRNA (50 ng) was injected to individual oocytes. At the 3rd day after injection oocytes were transferred to hypotonic solution (70 mOsm) and the images of oocytes were obtained by videomicroscopic technique at the indicated times.

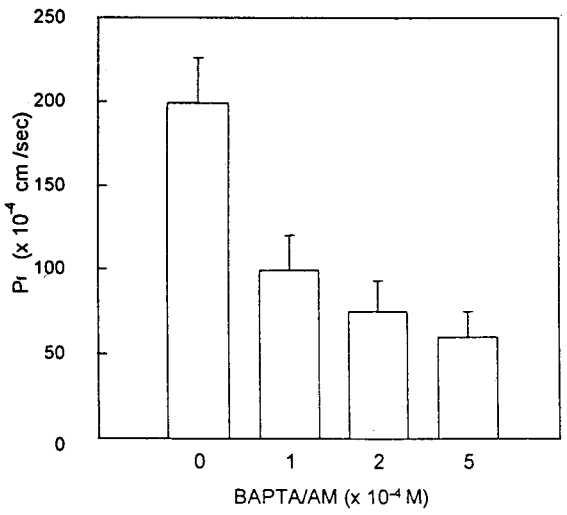

Fig. 8. Effect of BAPTA/AM on the osmotic water permeability of the AQP4 cRNA-injected oocytes. AQP4 cRNA-injected oocytes were pretreated with the indicated concentrations of BAPTA/AM for 1 hr. Osmotic water permeabiltiy was measured by the videomicroscopy described in the Methods and Materials. Data represent mean ± SEM. (n=5)

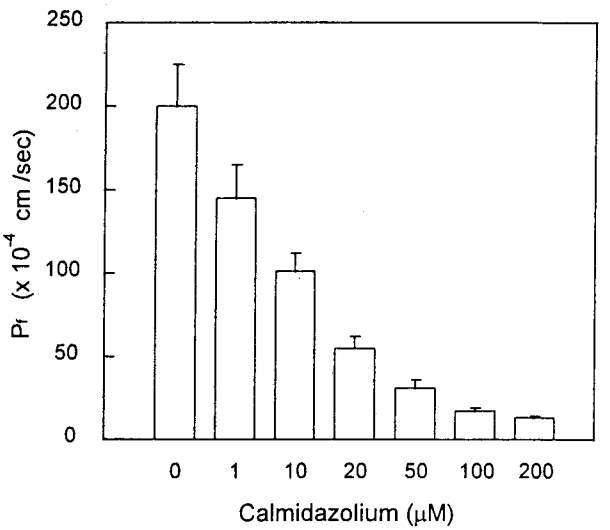

Fig. 9. Effect of calmidazolium on the osmotic water permeability of the AQP4 cRNA-injected oocytes. AQP4 cRNA-injected oocytes were pretreated with the indicated concentrations of calmidazolium for 30 min. Osmotic water permeabiltiy was measured by the videomicroscopy described in the Methods and Materials. Data represent mean±SEM. (n=5)

1 hr reperfusion after occlusion of MCA for 30 min and then recovered gradually by further reperfusion (Fig. 6).

Regulation of AQP4 water channel expressed in Xenopus oocytes

To test whether AQP4 water channels are regulated

by changes in intracellular Ca^{2+} levels, P_f in *Xenopus* oocytes was measured after injection with 50 nl of water or 50 nl of water containing 50 ng of AQP4 cRNA. Three days after injection, the oocytes were transferred from 200 to 70 mOsM modified Barth's buffer, and swelling was measured at 22°C. Water-injected control oocytes swelled minimally, whereas AQP4 RNA-injected oocytes swelled rapidly (Fig. 7)

To determine whether the osmotic permeability in AQP4-injected oocytes is regulated by changes in intracellular Ca^{2+} levels, the effect of pretreatment of BAPTA/AM was examined. Oocytes were pretreated with various concentrations of BAPTA/AM for 30 min in the Ca^{2+}-free Barth's solution. As shown in Fig. 8, the pretreatment of BAPTA/AM inhibited P_f in AQP4 injected oocytes from 0.1 to 0.5 mM as a dose-dependent manner, which suggests that decrease in intracellular Ca^{2+} levels inhibit P_f of AQP4 water channels.

To test whether the effect of changes in intracellular Ca^{2+} levels on P_f was mediated by Ca^{2+}/calmodulin kinase, effect of calmidazolium, a Ca^{2+}/calmodulin kinase inhibitor, was examined. AQP4- injected oocytes were pretreated with the indicated concentrations of calmidazolium for 30 min. Calmidazolium inhibited P_f of AQP4-injected oocytes from 10 μM to 200 μM as a dose-dependent manner (Fig. 9). However, 100 μM calmidazolium did not affect on P_f in AQP-CHIP-injected oocytes (data not shown).

DISCUSSION

This study showed that AQP4 expression in the brain is developmentally regulated and its expression rose slowly from E21 to PN1, then strongly at PN7, and then decreased. It has been reported that AQP4 RNA expression is not changed in the kidney after birth (Yamamoto et al, 1997), suggesting that AQP4 mRNA expression in various tissues may be differentially regulated during development. Differential expression in various tissues during development was also reported in the AQP-CHIP mRNA (Bondy et al, 1993). The data in this study showed that the AQP4 mRNA expresseion in specific areas of brain changed during development. In newborn rats, AQP4 mRNA was expressed strongly in ependymal cells of the ventricles. However, expression of AQP4 mRNA in glial cells surrounding supraoptic and paraventricular nuclei, cerebellum and hippocampus gradually increased and its expression in ependymal cells decreased in adult rats. Expression of AQP4 mRNA in the subfornical organ, which plays a major role in the control of fluid balance and arterial pressure (Johnson & Gross, 1993; McKinley et al, 1996), was detected at PN1 and was not changed remarkably thereafter. Recent immunocytochemical studies revealed that AQP4 pretein is present at astrocyte membrane domains facing blood vessels and pia and is heavily expressed in osmosensory areas of brain including hypothalamic magnocellular nuclei and the subfornical organ (Nielsen et al, 1997), suggesting that AQP4 could act as a transducer or amplifier in the osmoregulatory response. Therefore, the changes in AQP4 mRNA expression in osmosensory areas during brain development may reflect changes in relative contribution of these areas to body fluid homeostasis.

In this study cryogenic brain injury did not affect on AQP4 mRNA expression. Brain edema by cryogenic injury, which has been used as a model of contusive traumatic brain injury, is mainly caused by alterations in blood-brain barrier and/or vascular permeability (Trout et al, 1986; James & Schneider, 1990; Vinas et al, 1995), which may be related to no changes in AQP4 mRNA mRNA in crogenic injury. In contrast with cyrogenic brain injury, ischemic brain injury induced decreases in AQP4 mRNA expression. Although decrease in AQP4 mRNA by ischmia-reperfusion may be caused by nonspecific degradation of RNA, gradual recovery of AQP4 mRNA level after reperfusion suggests down regulation of AQP4 expression, because it has been known that neuronal injury progresses after reperfusion (Bazan et al, 1995; Kristian & Siesjo, 1996; Tymianski & Tator, 1996).

In this study we determined if AQP4 water channel could be regulated at protein phosphorylation. The pretreatment of BAPTA/AM and calmidazolium decreased P_f in AQP4-injected oocytes as a dose-dependent manner, indicating that activation of Ca^{2+}/Calmodulin kinase by increase in intracellular Ca^{2+} level increases the P_f of AQP4-injected oocytes. Nonspecific effects of the pretreatment of BAPTA/AM or calmidazolium could be excluded by the data that P_f in AQP-CHIP-injected oocytes was not affected on the pretreatment of calmidazolium. In global or forebrain ischemia, Ca^{2+} influx through

channels gated by voltage-dependent Ca^{2+} channels or glutamate receptors triggers reactions that limit the survival of neurons during reperfusion, leading to secondary neuronal death after hours or days of survival (Bazan et al, 1995; Kristian & Siesjo, 1996; Tymianski & Tator, 1996). It is interesting that increase of P_f through AQP4 water channel by changes in intracellular Ca^{2+} levels can be compensated by decrease in expression of AQP4 mRNA in brain ischemia. The different regulations of AQP4 function at protein and transcription levels by brain ischemia may reflect a homeostatic control of AQP4 function at various pathophysiologic conditions.

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